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Investigating the Neurobiological Basis  
Underlying the Sex-Specific Production of  
Courtship Song in *Drosophila*: The Roles of Sex  
Determination Genes *fruitless* and *doublesex*

A thesis submitted for the degree of Doctor of Philosophy at the University of  
Glasgow

By

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## Abstract

The elaborate courtship ritual performed by *Drosophila melanogaster* males to interest females in copulation is used as a model to investigate the genetic and neurobiological basis for the specification of complex behaviours. One component of this courtship ritual is the ‘lovesong’, which both promotes mating and carries vital species-specific information. Sex mosaic studies have shown that specific regions of the central nervous system (CNS) must be male in order to produce this courtship song. Indeed, two genes in the sex determination hierarchy, *fruitless* (*fru*) and *doublesex* (*dsx*), which are responsible for sexual differentiation in both the CNS and non-neuronal tissues, are required for wild-type song production. However, the critical differences underlying the sexually dimorphic production of courtship song are unknown; moreover, the relative contributions of *fru* and *dsx* to the generation of these differences are not known.

The central aim of this thesis was to investigate the neurobiological basis for the sexually dimorphic production of courtship song; and in addition, to determine the individual and combined contributions of *fru* and *dsx* in creating these essential differences. The long-term goal of this study was to determine the mechanism by which genetic factors such as *fru* and *dsx* can create the potential for courtship song by specifying aspects of CNS development and function. As a first step towards this long-term goal, the work presented in this thesis has identified the individual and combined contributions of *fru* and *dsx* to the production of courtship song. Moreover, a requirement for both *fru* and *dsx* in the specification of a neural substrate underlying courtship song was shown.

Using females constitutively expressing the male-specific *fru* isoforms, the individual contribution of *fru* to the production of courtship song was determined; and the amount and quality of song generated by these females was found to be highly aberrant. Thus *fru* alone does not specify courtship song production. In fact, wild-type courtship song was only achieved when females expressed the male-specific isoforms of both *fru* and *dsx*, demonstrating that both genes are required for the specification of courtship song.

Next, the co-expression of *fru* and *dsx* was examined, where *fru* and *dsx* were found to be co-expressed in three distinct regions of the CNS. One of these regions, the mesothoracic ganglion (Msg), is strongly implicated in the production of courtship song, suggesting that *fru* and *dsx* may act in concert to specify courtship song production. A closer examination of this region revealed a male-specific population of *fru*-expressing neurons in the Msg. This population of neurons was not present in females constitutively expressing *fru*'s male-specific isoforms, demonstrating that *fru* alone does not specify the development of a sexually dimorphic Msg. Instead, a critical requirement for *dsx*, alongside *fru*, in the specification of this sexually dimorphic population of neurons was shown. Thus a critical requirement for both *fru* and *dsx* in the specification of courtship song, and in the creation of a sexually dimorphic population of neurons in the Msg has been revealed.

Finally, to determine how this sexually dimorphic population of neurons is linked to courtship song production, this thesis described the generation of genetic tools to allow the visualization and manipulation of these sex-specific neurons. Using the UAS/GAL4 system of targeted gene expression, another sexually dimorphic population of neurons was identified in the posterior brain, which is, like the Msg, a region where *fru* and *dsx* are co-expressed. Together, these results suggest that *fru* and *dsx* act to generate sexually dimorphic populations of neurons in regions of co-expression, where these neurons may form part of the sex-specific neural circuits underlying the performance of sexually dimorphic behaviours.

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## List of Accompanying Materials

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## **Author's declaration**

I hereby declare that all the work reported in this thesis is my own unless stated otherwise in the text. None of the work has been previously submitted for any other degree at any other institution. All sources of information used in the preparation of this thesis are indicated by reference.

# Glossary

## Anatomical Terms

Abdominal Ganglion	Abg
Accessory Gland	AG
Antennal lobe	AL
Basalar Muscle 1	B1
Basalar Muscle 2	B2
Basalar Muscle 3	B3
Basalar Muscle 4	B4
Central nervous system	CNS
Common oviduct	cOD
Direct flight muscles	DFM
Ejaculatory bulb	EjB
Ejaculatory duct	EjD
Fat tissue	FT
First axillary muscle	AX1a
Lateral oviduct	lOD
Male-specific somatic gonadal precursors	msSGP
Mesothoracic ganglion	Msg
Motor neurons innervating the DFM	mnDFM
Muscle of Lawrence	MOL
Mushroom bodies	MB
Neuromuscular junction	NMJ
Olfactory receptor neuron	ORN
Seminal vesicle	SV
Spermatheca	ST
Sternobasalar muscle	SB
Testicular duct	TD
Third Axillary muscle	AX3a
Uterus	U
Vagina	V
Vas deferens	VD
Ventral nerve cord	VNC

## Genetic Terms

<i>Abdominal-A</i>	<i>AbdA</i>
<i>Antennapedia</i>	<i>Antp</i>
<i>β-Galactosidase (gene)</i>	<i>lacZ</i>
<i>β-Galactosidase (protein)</i>	<i>β-Gal</i>
<i>apterous</i>	<i>ap</i>
<i>cacophony</i>	<i>cac</i>
<i>Canton S</i>	<i>CS</i>
<i>Curly of Oster</i>	<i>CyO</i>
<i>decapentaplegic</i>	<i>dpp</i>
<i>doublesex</i>	<i>dsx</i>
<i>ebony</i>	<i>e</i>
<i>embryonic lethal, abnormal vision</i>	<i>elav</i>
<i>(enhanced) Green Fluorescent Protein</i>	<i>(e)GFP</i>



Flippase site-specific recombinase	FLP
Flippase recognition site	FRT
<i>fruitless</i>	<i>fru</i>
<i>γ-glutamyl carboxylase</i>	GC
<i>grainyhead</i>	<i>grh</i>
<i>grim</i>	<i>grim</i>
<i>Gustatory receptor gene</i>	<i>Gr</i>
<i>head involution defective</i>	<i>hid</i>
<i>Humeral</i>	<i>Hu</i>
<i>inactive</i>	<i>iav</i>
<i>intersex</i>	<i>ix</i>
Mosaic analysis with a repressible cell marker	MARCM
Membrane-bound mouse lymphocyte marker CD8	mCD8
<i>mini-white</i>	<i>mw</i>
<i>Neural Lazarillo</i>	<i>NLaz</i>
<i>no-on-transientA</i>	<i>nonA</i>
<i>Olfactory receptor gene</i>	<i>Or</i>
<i>pugilist</i>	<i>pug</i>
<i>period</i>	<i>per</i>
Polycomb response element	PRE
<i>reaper</i>	<i>rpr</i>
<i>reversed polarity</i>	<i>repo</i>
<i>rosy</i>	<i>ry</i>
<i>Serrate</i>	<i>Ser</i>
<i>Sex-lethal</i>	<i>Sxl</i>
<i>shibire</i>	<i>shi</i>
<u>Stable</u> <u>insulated</u> <u>nuclear enhanced</u> <u>GFP</u> .	Stinger
<i>Stubble</i>	<i>Sb</i>
<i>suppressor-of-hairy-wing</i>	<i>su(Hw)</i>
SV40 Transcript Terminator	SV40
T4 bacteriophage	T4
<i>transformer</i>	<i>tra</i>
<i>transformer-2</i>	<i>tra-2</i>
<i>Tubby</i>	<i>Tb</i>
<i>tyramine-β-hydroxylase</i>	<i>TβH</i>
<i>Tyrosine decarboxylase-2</i>	<i>Tdc2</i>
<i>Ultrabithorax</i>	<i>Ubx</i>
Upstream Activating Sequence	UAS
<i>white</i>	<i>w</i>
Yeast Transcriptional Activator GAL4	GAL4
<i>yolk-protein-1 and -2</i>	<i>yp-1, yp-2</i>

## Measurements

Acidity [-log <sub>10</sub> (Molar concentration of H <sup>+</sup> ions)]	pH
Base pair	bp
Centimetre	cm
Centrifugal force equal to gravitational acceleration	g
Decibel	dB
Degrees Centigrade	°C
Gram	g
Hour	hr
Kilobases	kb
Kilovolt	kV

Litre	L
Melting temperature	T <sub>m</sub>
Metre	m
Microgram	μg
Microlitre	μL
Micrometre	μm
Micromolar	μM
Milligram	mg
Millilitre	mL
Millimetre	mm
Millimolar	mM
Millisecond	ms
Millivolt	mV
Minute	min
Molar	M
Nanogram	ng
Nanometre	nm
Optical density	O.D.
Percent	%
Picogram	pg
Second	sec or s
Volt	V
Volume per volume	v/v
Weight per volume	w/v

### Behavioural Parameters

Courtship index	CI
Cycles per pulse	CPP
Interpulse interval	IPI
Mean pulses per train	MPPT
Pulse trains per minute	PTPM
Song index	SI
Song bouts per Minute	SBPM
Wing extension index	WEI

### Chemicals

1-ethyl-3(3-(dimethylamino)propyl) carbodiimide	EDAC
2' Deoxyribonucleic acid	DNA
5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside	X-gal
Bovine serum albumin	BSA
Bromodeoxyuridine	BrdU
Carbon dioxide	CO <sub>2</sub>
Complementary DNA	cDNA
Dimethyl formamide	DMF
Ethanol	EtOH
Ethidium Bromide	EtBr
Ethylene diamine tetraacetic acid	EDTA
Ethylene glycol tetraacetic acid	EGTA
Horseradish peroxidase	HRP
Hydrochloric acid	HCl
Isopropyl-β-D-thiogalactopyranoside	IPTG

Messenger RNA	mRNA
Phosphate buffered saline	PBS
PBS with Triton-X	PBT
PBS with Triton-X and 5% SNS	PTN
PBS with Triton-X and 0.1% BSA	PAT
Ribonucleic acid	RNA
Sheep normal serum	SNS
Sodium chloride	NaCl
Sodium dodecyl sulphate	SDS
Sodium hydroxide	NaOH
Tetramethylrhodamine B isothiocyanate	TRITC
Transfer RNA	tRNA

## Computing and Statistical Terms

Adobe illustrator file	ai
Analysis of variance	ANOVA
Digital video file	dv
Liquid crystal display	LCD
Look up table	LUT
Quicktime movie file	mov
Personal computer	PC
Portable document format	pdf
Tagged image file format	tiff
Standard deviation	s.d.
Standard error of the mean	s.e.m.

## Miscellaneous

7-tricosene	7-T
7,11-heptacosadiene	7,11-HD
7,11-nonacosadiene	7,11-ND
11- <i>cis</i> -vacacenyl acetate	cVA
Bacterial artificial chromosome	BAC
Broad-complex/Tramtrack/Bric-à-brac	BTB
Cuticular hydrocarbon	CH
Differential interference contrast	DIC
Ganglion mother cell	GMC
Inverted repeats	IR
Neuroblast	NB
Overnight	O/N
Polyacrylamide gel electrophoresis	PAGE
Polymerase chain reaction	PCR
Ribonuclease A	RNaseA
<i>Drosophila</i> Schneider 2 Cells	S2 Cells
<i>P</i> transposable element	<i>P</i> -element
Ultraviolet	UV

## 1 Introduction

## 1.1 Genes, Brain and Behaviour

Behaviour is defined as a reaction or response to an external or internal stimulus, where the behavioural repertoire of an organism reflects the unique selection pressures found in its immediate environment. Indeed, diverse behavioural solutions have been found by individual species to meet the particular demands of their respective habitats. However, one challenge common to all species, regardless of habitat, is the need to reproduce.

Reproduction in most organisms requires that an individual be able to both identify a suitable mate, and to perform the necessary steps to ensure that reproduction occurs as quickly as possible. Thus successful reproduction requires the ability to detect the particular combination of visual, olfactory or auditory cues that are transmitted by conspecific individuals, and that these cues elicit an appropriate behavioural response. A further level of complexity is the often sexually dimorphic nature of these reproductive behaviours. To minimize the possibility of rejection, these responses must be consistent not only between individuals of the same species, but individuals of the same sex. How is this consistency achieved?

Given that the behavioural repertoire of an individual often reflects the environment in which they are found, selection may operate on the behaviour of individuals, where small changes in the genome resulting in advantageous behaviours are transmitted to the next generation, and over time, dispersed throughout a population of individuals (Bate, 1998). Therefore, aspects of behaviour may be represented in the genome, where interacting networks of genes specify the development and functional properties of the CNS, giving each individual the potential to perform these most vital behaviours in response to specific cues (Sokolowski, 2001). These most essential behaviours are called innate behaviours, and require no prior experience of a stimulus in order to elicit the appropriate behavioural response. In the case of sexual and reproductive behaviours, then, the cues to elicit particular behaviours may be sex-specific, and as a result, the development and function of the CNS may also be dimorphic. Therefore, investigating how genes control the sexually dimorphic development and function of the CNS will give insight into the mechanism by which two morphologically and behaviourally distinct sexes are generated, and

in addition, contribute to the current understanding of the genetic regulation of developmental pathways (Schütt and Nothiger, 2000).

### **1.1.1 Studying Genes, Brain and Behaviour**

Although in the broadest sense behaviour is defined as a reaction or response to an internal or external stimulus, for the purposes of scientific investigation, a specific behaviour must be defined in the context of its own paradigm (reviewed by Sokolowski, 2001). Once a given behaviour has been defined in this way, the behaviour of wild-type individuals of the same age and experience can be quantified, and subsequently compared to individuals that have undergone some type of genetic manipulation (Sokolowski, 2001). Eventually, genes involved in the development and function of neural circuits underlying specific behaviours can be identified. Thus, investigating the genetic and neurobiological basis of sexual and reproductive behaviours requires a genetically tractable model organism with easily quantifiable reproductive behaviours.

### **1.1.2 *Drosophila* as a Model Organism to Study Sexual Behaviour**

Genetic tools such as visible genetic markers, balancer chromosomes, deletions covering large portions of the genome, and *P*-element transgenesis make *Drosophila melanogaster* the model organism of choice in the investigation of the genetic and neurobiological basis of behaviour. Combined with a fully sequenced genome (Adams *et al.*, 2000) and recently developed technology to target specific genes by homologous recombination (Rong and Golic, 2000; Rong *et al.*, 2002; reviewed by Bi and Rong, 2003), these genetic tools allow both forward and reverse genetic screens to be performed in *Drosophila*, greatly simplifying the identification of single genes involved in the development and function of neural circuits controlling specific behaviours.

In addition to the numerous genetic tools, the sexual behaviour of *Drosophila melanogaster* has been intensively studied. The elaborate courtship ritual performed by males is easily quantified, shows little intra-strain variation, and is one of the most complex behaviours performed by flies (reviewed by Villella and Hall, 2007; Billeter *et al.*, 2006a). However, the most significant advantage of studying courtship behaviour in *Drosophila* is the sexually dimorphic nature of

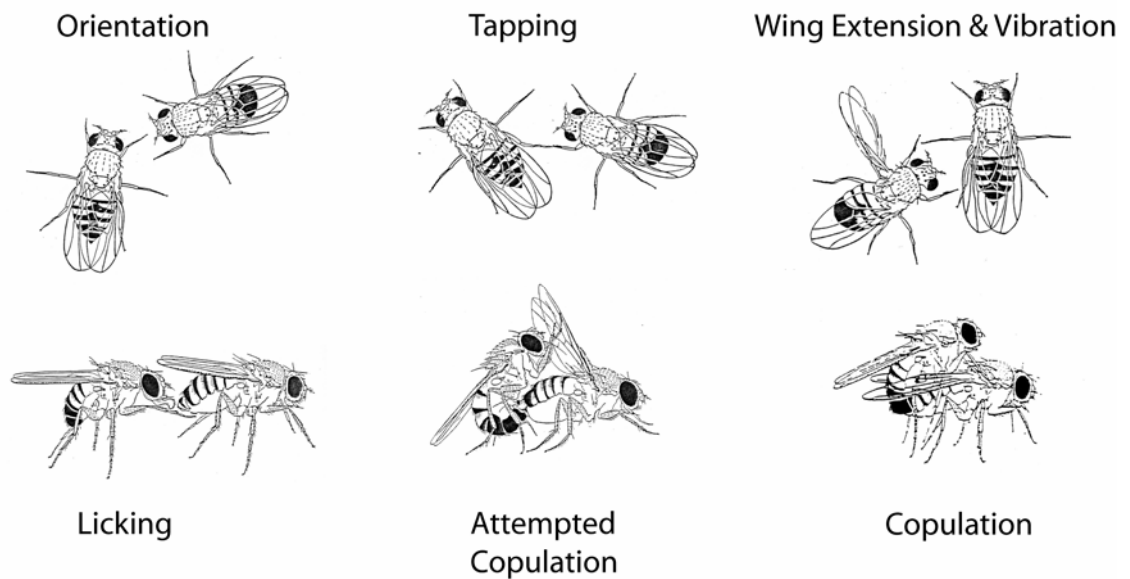
the behaviour, where two naturally occurring behavioural variants exist: males and females. Many studies have investigated the genetic basis for the creation of two sexes, and genes involved in the sex-specific development and function of the CNS have been identified; demonstrating the power of *Drosophila* in the investigation of genes controlling the development and function of neural circuits underlying specific behaviours (reviewed by Billeter *et al.*, 2002; Billeter *et al.*, 2006a; Villella and Hall, 2007).

### 1.1.2.1 Courtship Behaviour

Courtship behaviour in *Drosophila* is a complex behaviour performed by the male, with the sole purpose of convincing the female to copulate with him (reviewed by Hall, 1994). It consists of the following stereotypical sequence of behaviours: following, orientation, tapping, wing extension, courtship song production, licking, attempted copulation, and finally, if successful, copulation (Bastock and Manning, 1955) (Figure 1.1). Although female sexual behaviour primarily consists mostly of more subtle behaviours, such as running away, copulation will not occur until sufficient stimulation has been given by the male to increase the receptivity of the female, indicated by the female slowing down, and opening her genital plates for copulation. During copulation, a complex mixture of sperm and seminal fluids are transferred to the female, resulting in fertilization and a change in behaviour, respectively (reviewed by Wolfner, 1997; Kubli, 2003). These behavioural changes include a loss of receptivity to the advances of other males, an increase in egg laying, and a decrease in locomotion compared to virgin females (reviewed by Wolfner, 1997; Kubli, 2003). In addition, the pattern of pheromone synthesis is altered to include compounds inhibitory to male courtship (Manning, 1967a; reviewed by Greenspan and Ferveur, 2000). If the female is unreceptive due to recent mating, she will reject the male's advances by repeatedly flicking her wings, kicking the male in the head with her hind legs, or extruding her ovipositor (Hihara, 1981; Spieth and Ringo, 1983).

Although the courtship ritual itself may only last for a short period of time prior to copulation, critical sensory cues are exchanged between the sexes at this time, which stimulate both the male and female, and convey vital species-

specific information (reviewed by Greenspan and Ferveur, 2000; Billeter *et al.*, 2002; Billeter *et al.*, 2006a).



**Figure 1.1**-The Courtship Ritual of *Drosophila melanogaster*. The male fly performs an elaborate series of behaviours to convince the female to copulate with him, where a number of sensory cues are exchanged by the sexes during this courtship ritual. Courtship includes the following steps: orientation, tapping, wing extension and vibration (to generate courtship song), licking, attempted copulation, and finally, copulation (original figure from Burnet and Connolly, 1974).

### 1.1.2.2 Sensory Cues Exchanged During Courtship Behaviour

The sensory cues transferred during each step of the courtship ritual act on a number of sensory modalities, including the visual system, the chemosensory system and the mechanosensory system; and consist of both stimulatory and/or inhibitory cues (reviewed by Greenspan and Ferveur, 2000; Billeter *et al.*, 2002; Billeter *et al.*, 2006a). Together, these cues ensure that each party receives sufficient stimulation, and that an appropriate mate is chosen. The major sensory signals and their importance to the courtship ritual are discussed below.

#### The Visual System

Visual cues are critical to the male's identification of a potential mate, and are among the first to be exchanged between the sexes (Connolly *et al.*, 1969; Tompkins *et al.*, 1982). In fact, studies have shown that female movement and shape alone can elicit the male to perform courtship behaviour (Connolly *et al.*, 1969; Tompkins *et al.*, 1982). Following the initial identification of a suitable target by the male, visual cues continue to play an important role in tracking



behaviour, where the male monitors the position and movements of the female, and keeps in relatively close proximity (Cook, 1979; Cook, 1980).

Visually impaired males are able to perform all steps of the courtship ritual (Gailey *et al.*, 1986; Joiner and Griffith, 1997); however, the importance of visual cues in courtship behaviour is demonstrated both by the overall reduction in the amount of courtship behaviour performed by a visually impaired male, and by the reduction in courtship success (Geer and Green, 1962; Grossfield, 1966; Connolly *et al.*, 1969; DeJianne *et al.*, 1981; Tompkins *et al.*, 1982; Markow, 1987; Chatterjee and Singh, 1988; Stocker and Gendre, 1989; Joiner and Griffith, 1997).

### The Chemosensory System

Upon visual identification of an appropriate target, the male will follow the target female, orient to her, and then proceed to tap the abdomen of the female with his foreleg. These early courtship steps will then be followed by the later stages of wing extension and vibration, licking and attempted copulation. Chemosensory cues are transferred between the sexes over short distances while the male is following and orienting, and by physical contact, during tapping and licking (reviewed by Stocker, 1994; Billeter *et al.*, 2002; Ferveur, 2005; Billeter *et al.*, 2006a). Chemosensory cues come in two forms: gustatory, which are received by specialized bristles located on the foreleg and the proboscis; and olfactory, which are received by specialized hairs called sensillae located on the antennae and maxillary palps (reviewed by Stocker, 1994; Ferveur, 2005; Vosshall and Stocker, 2007). These cues can be either excitatory or inhibitory, and modulate both the amount and the target of courtship behaviour performed by a male (reviewed by Ferveur, 2005).

In *Drosophila melanogaster*, the cuticular hydrocarbons (CH) that act as sex pheromones are synthesized in a sexually dimorphic manner. The predominant female CHs are 7,11-heptacosadiene (7,11-HD) and 7,11-nonacosadiene (7,11-ND) (reviewed by Jallon, 1984; Ferveur, 2005). These pheromones have a stimulatory effect on males during courtship; indeed, studies have shown that female sex pheromones can induce wing vibration in males (Antony and Jallon, 1982; Jallon, 1984; Antony *et al.*, 1985). The importance of this stimulation is demonstrated by the fact that females lacking 7,11-HD and 7,11-ND are less

‘attractive’ to males (Savarit *et al.*, 1999), and also that males with olfactory impairments perform less courtship towards target females (Gailey *et al.*, 1986; Stocker and Gendre, 1989; Joiner and Griffith, 1997; reviewed by Greenspan and Ferveur, 2000).

Although the stimulatory effect of female-specific pheromones 7,11-HD and 7,11-ND on males is well documented, the anti-aphrodisiac effects of the male CH, 7-tricosene (7-T), are also important to mate recognition (Scott, 1986; Ferveur and Sureau, 1996). Males without 7-T display elevated levels of intermale courtship, demonstrating an important role for 7-T in suppressing courtship towards inappropriate targets (Scott, 1986; Ferveur and Sureau, 1996). Likewise, the inability of genetically modified males to detect another male-specific pheromone, 11-*cis*-vacacenyl acetate (cVa), has been found to induce inappropriately high levels of intermale courtship (Kurtovic *et al.*, 2007). Thus inhibitory cues are just as important as stimulatory cues in recognizing a suitable mate.

Clearly, chemosensory cues play an important role in stimulating or inhibiting courtship between the sexes; however, an additional role for these chemosensory cues is the identification of conspecific individuals (reviewed by Ferveur, 2005). Normally, visual cues will attract a male to a potential mate, and the male will begin the early stages of courtship. If the pheromones are typical of a conspecific female, courtship will continue, however, if pheromones typical of another species are detected, courtship will stop at this point (Savarit *et al.*, 1999). Thus chemosensory cues modulate the amount and target of courtship behaviour performed by a male by transmitting vital information about the sex and the species of the potential target.

### The Mechanosensory System

Acoustic communication in *Drosophila melanogaster* will be discussed in detail in section 1.3. Briefly, the ‘lovesong’ is an important mechanosensory cue during courtship, and is produced when the male extends and vibrates his wing (Ewing and Bennet-Clark, 1968). Both sexes can be stimulated by courtship song, and it communicates critical species-specific information (von Schilcher, 1976a; Kyriacou and Hall, 1982; Kyriacou and Hall, 1984; Kyriacou and Hall, 1986; Eberl *et al.*, 1997; Ritchie *et al.*, 1999; Rybak *et al.*, 2002). The importance of song to

the courtship ritual is demonstrated by the fact that wingless males are more successful when artificial song pulses are played back during courtship than wingless males where no song is played back (Bennet-Clark and Ewing, 1969).

A unique combination of species- and sex-specific cues exchanged by the sexes ensures the efficient progression from courtship to copulation. This progression demands that these sexually dimorphic cues be detected, and that they elicit a consistent behavioural response; therefore, in the next section, the genetic basis for the elicitation and performance of these sexually dimorphic behaviours will be discussed.

## 1.2 Genetic Basis of Courtship Behaviour

Courtship behaviour in *Drosophila melanogaster* is an excellent model to investigate the genetic basis of innate behaviours for a number of reasons. First, courtship behaviour is sexually dimorphic, and genes have been identified which are involved in generating these dimorphic behaviours. Second, given that courtship is itself composed of a sequence of behaviours, genes can be identified which control the development of neural circuits both underlying individual behaviours, and those involved in co-ordinating the performance of each behaviour in sequence (Markow, 1987; Manoli and Baker, 2004; Billeter *et al.*, 2006b). Finally, the diversity of sensory inputs carrying vital sex- and species-specific information allows the investigation of how sensory cues are received, processed, and translated into an appropriate behavioural response. Therefore, by investigating the genetic basis for courtship behaviour in *Drosophila*, a greater understanding of the mechanism by which sex-specific behaviours are programmed into the CNS will be gained.

Mutations in many genes have been shown to affect the amount and quality of courtship behaviour performed by the male fly. Generally speaking, mutations that affect courtship behaviour fall into two classes: 1) mutations in genes which also have affect the performance of other behaviours, and 2) mutations in genes responsible for determining the sexual identity of the fly. Only the latter class of mutations will allow the identification of genes responsible for controlling the sexually dimorphic development and function of the CNS, and permit an investigation into how sexually dimorphic behaviours are generated. Thus, in the

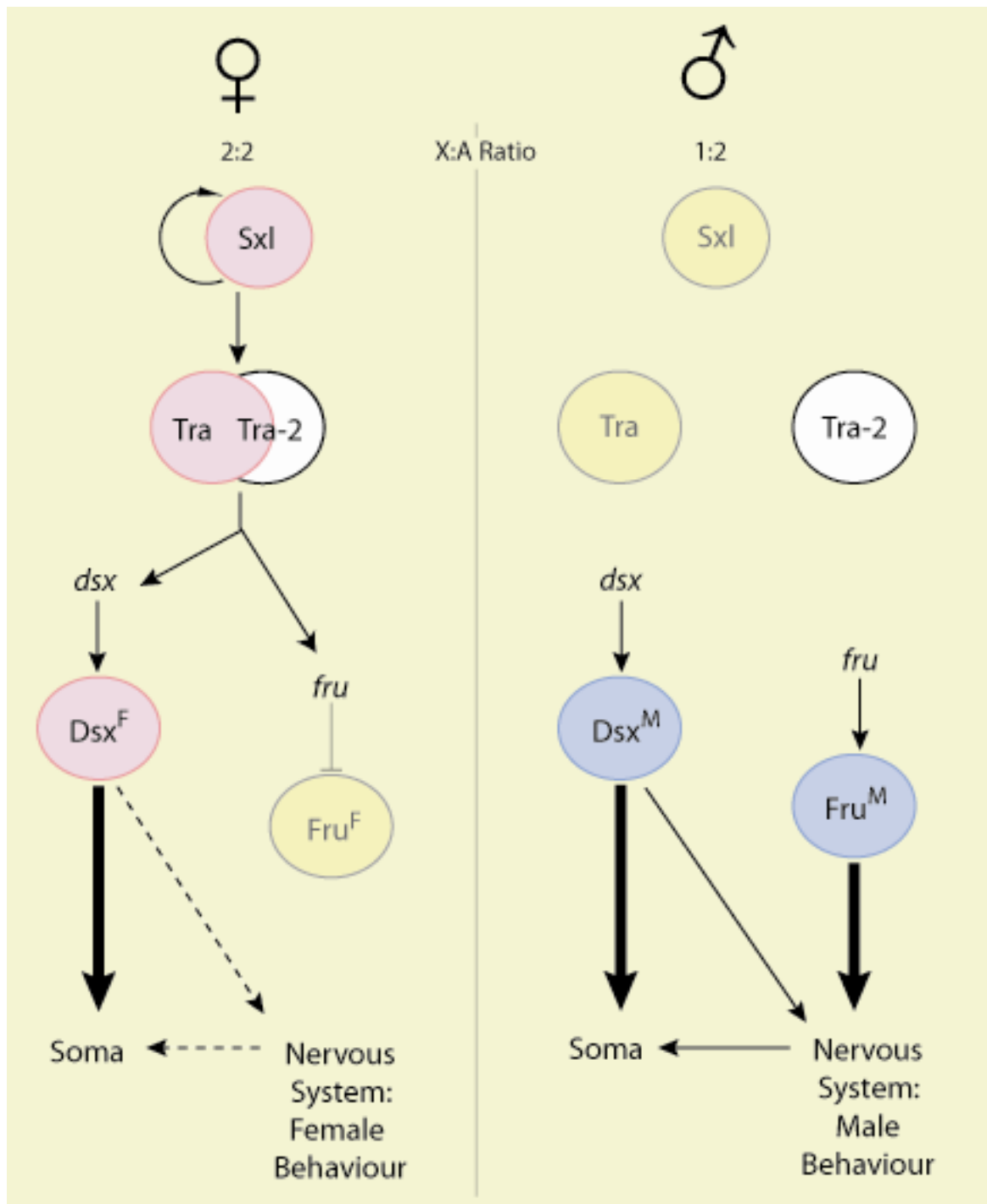
following sections, the genes known to be involved in sexual differentiation and the specification of sexual behaviour in *Drosophila* will be discussed.

### 1.2.1 Sex Determination in *Drosophila*

In *Drosophila melanogaster*, the ratio of sex chromosomes to autosomes initiates a cascade of sex-specific splicing, ultimately resulting in the differentiation of either male- or female-specific sexual characteristics and behaviour (Figure 1.2). Given that courtship is a sexually dimorphic behaviour, it is not surprising that mutations in genes in this sex determination pathway have striking effects on courtship behaviour (McRobert and Tompkins, 1985; reviewed by Billeter *et al.*, 2002; Billeter *et al.*, 2006a; Villella and Hall, 2007).

Briefly, the X:A ratio controls the activity state of *Sex-lethal* (*Sxl*), a *cis*-acting RNA splicing factor. When the X:A ratio is 1, as in females, *Sxl* is activated, and a functional *Sxl* protein is produced (Cline, 1984). The primary target of *Sxl* is *transformer* (*tra*) pre-mRNA, where *Sxl* binds and causes a sex-specific splice, resulting in a functional *Tra* protein (McKeown *et al.*, 1987; Boggs *et al.*, 1987; Nagoshi *et al.*, 1988; Inoue *et al.*, 1990). *Tra*, an RNA splicing factor, in conjunction with the non-sex-specific splice factor Transformer-2 (*Tra-2*), goes on to cause a sex-specific splice in each of two downstream genes in the sex determination pathway: *fruitless* (*fru*) and *doublesex* (*dsx*) (Ryner *et al.*, 1996; Heinrichs *et al.*, 1998; Nagoshi *et al.*, 1988; Hoshijima *et al.*, 1991; Inoue *et al.*, 1992), where each gene sits atop an independent branch of the sex determination pathway, described in further detail below. In males, with an X:A ratio of 0.5, *Sxl* is repressed, and a functional *Sxl* protein is not made (Cline, 1984). Thus, in males, no active *Tra* is present, and, in the absence of functional *Tra*, *fru* and *dsx* each undergo a default splice (Ryner *et al.*, 1996; Nagoshi *et al.*, 1988).

Together, the genes in this sex determination pathway control most aspects of sexual morphology and behaviour (reviewed by Baker *et al.*, 2001; Billeter *et al.*, 2002; Billeter *et al.*, 2006a); however, *fru* and *dsx*, the genes downstream of *tra*, make distinct contributions to the specification of sexual morphology and/or behaviour.



**Figure 1.2**-Sex Determination in *Drosophila melanogaster*. In females, the ratio of sex chromosomes to autosomes (X:A) is 2:2, resulting in an active Sxl protein. Sxl causes a sex-specific splice in *tra* mRNA, resulting in a functional Tra protein. Tra, in conjunction with non-sex-specific Tra-2, causes a sex-specific splice in both *dsx* and *fru* mRNA, respectively, resulting in the production of the female-specific isoform of *dsx*, Dsx<sup>F</sup>, and the introduction of a stop codon into the *fru* transcript, where no *fru* protein is made in females. In males, the X:A ratio is 1:2, and no active Sxl is made, and as a result, no functional Tra is made, and both *dsx* and *fru* undergo a default splice to generate the male-specific isoforms of both proteins, Dsx<sup>M</sup> and Fru<sup>M</sup>. From Billeter *et al.* (2006a).

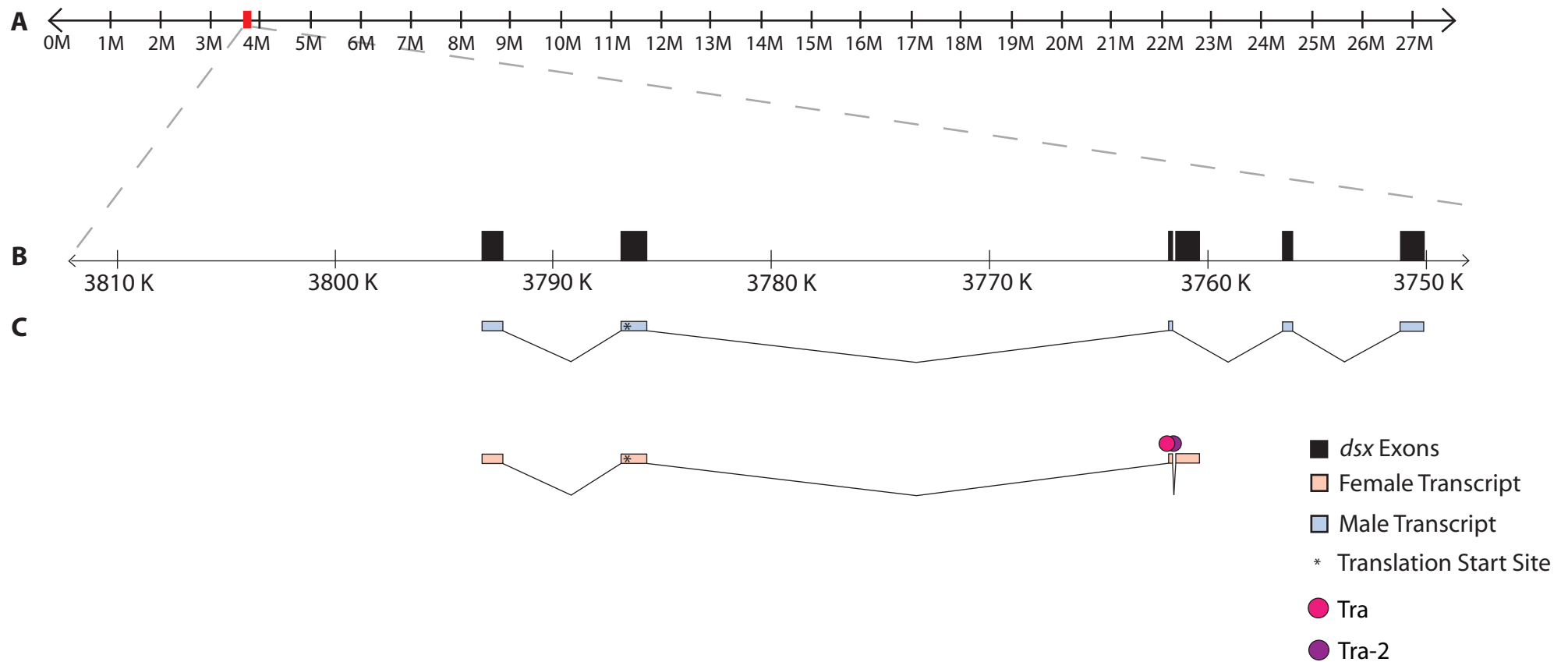
## **1.2.2 doublesex Controls Sexually Dimorphic Morphology and Biochemistry**

A mutation in the *dsx* locus was originally described by Hildreth (1965), which transformed both males and female into intersexes. Since, the molecular properties of *dsx* have been reported, along with its contribution to sexual differentiation and courtship behaviour.

### **1.2.2.1 Generating Sex-Specific *dsx* Isoforms**

The *dsx* locus spans approximately 43 kb, from cytogenetic location 84E5 to 84E6 (Figure 1.3), and encodes two related transcription factors, each containing an amino-terminus DM DNA-binding domain, and a carboxy-terminus protein interaction domain (Burtis and Baker, 1989; Erdman and Burtis, 1993; Coschigano and Wensink, 1993; An and Wensink, 1995).

The polypeptides encoded by *dsx* are sex-specific, and are generated by alternative splicing at the 3' end of the pre-mRNA (Burtis and Baker, 1989; Ryner and Baker, 1991). In females, the female-specific splice factor Tra, in conjunction with non-sex-specific Tra-2, causes a sex-specific splice at the 3' end of the transcript, resulting in the female-specific isoform of *dsx*, Dsx<sup>F</sup> (Nagoshi *et al.*, 1988; Hoshijima *et al.*, 1991; Inoue *et al.*, 1992). In males, who express no functional Tra, a default 3' splice site is used to produce the male-specific isoform of *dsx*, Dsx<sup>M</sup>. Thus the first 397 amino acids of Dsx<sup>M</sup> and Dsx<sup>F</sup>, which contain the DNA-binding DM domain, are identical, suggesting that Dsx<sup>M</sup> and Dsx<sup>F</sup> bind to the same transcriptional targets (Burtis and Baker, 1989; Erdman and Burtis, 1993). The carboxy terminus of the protein, on the other hand, which contains a protein interaction domain, differs between the isoforms, and is thought to mediate the interactions that allow each isoform to have such distinct effects on downstream targets (Burtis and Baker, 1989; Coschigano and Wensink, 1993; An and Wensink, 1995).



**Figure 1.3**-Schematic Representation of the *dsx* Locus. (A) Right arm of the third chromosome is shown, with the *dsx* locus indicated by a red box. M = megabases. (B) The *dsx* locus spans approximately 43 kb on the right arm of the third chromosome, from cytogenetic location 84E5-84E6, and has both sex-specific and non-sex-specific exons. (C) Transcripts from the *dsx* locus are sex-specifically spliced at the 3' end by the female-specific splice factor Tra in conjunction with non-sex-specific splice factor Tra-2.

### 1.2.2.2 *dsx* Controls External and Internal Sexual Characteristics

Male and female *Drosophila* are distinguished by a number of external and internal sexual characteristics (Ferris, 1950), including sexually dimorphic external genitalia, sex-specific abdominal pigmentation, the differentiation male- or female-specific internal reproductive organs, the development of male-specific bristles on the foreleg (sex combs), and the synthesis of sexually dimorphic pheromones (McRobert and Tompkins, 1985; Jallon *et al.*, 1988). Since the isolation of the first *dsx* mutant by Hildreth, (1965), many more *dsx* mutants have been reported, and the role of *dsx* in the process of sexual differentiation has been investigated in great detail.

Complete loss of function alleles in *dsx* cause both males and females to differentiate as intersexes (Hildreth, 1965). These intersexes develop with male-like pigmentation, can have either (or both) male or female internal reproductive organs, and have reduced sex combs on the foreleg. In addition, synthesis of the predominant female pheromones 7,11-HD and 7,11-ND has been shown to be under the control of the *dsx* locus in females (Jallon *et al.*, 1988; Waterbury *et al.*, 1999). The intersexual phenotypes are due to the loss of both positive and negative regulatory events mediated by the male- and female-specific isoforms of *dsx* (Hildreth, 1965; Burtis and Baker, 1989; Jursnich and Burtis, 1993; Waterbury *et al.*, 1999). For example, the ectopic expression of  $Dsx^M$  in either sex results in the transformation of leg bristles towards a sex comb-like morphology, and in addition, rescues the intersexual appearance of sex combs in *dsx*<sup>1</sup> mutants (Jursnich and Burtis, 1993). Moreover, females carrying a dominant *dsx* mutation, *dsx*<sup>Dom</sup>, in which  $Dsx^M$  is constitutively expressed, differentiate as morphologically male (Baker and Wolfner, 1988; Nagoshi and Baker, 1990). Similarly, the expression of  $Dsx^F$  has been shown to induce the expression of female-specific pheromones in males, and is sufficient for the development of female internal reproductive organs, abdominal pigmentation and external sexual characteristics (Burtis and Baker, 1989; Szabad and Nothiger, 1992; Waterbury *et al.*, 1999). Thus *dsx* controls most aspects of sexually dimorphic differentiation.



### 1.2.2.3 *dsx* at the Molecular Level

Although *dsx* is known to regulate most aspects of sexual differentiation, and has been shown to either directly or indirectly regulate a number of genes, the only downstream targets of *dsx* that have been confirmed at the molecular level are the *yolk protein* family of genes (Burtis *et al.*, 1991; Coschigano and Wensink, 1993; An and Wensink, 1995). Both Dsx<sup>M</sup> and Dsx<sup>F</sup> bind to a consensus sequence on the promoter of the *yolk protein* genes (Burtis *et al.*, 1991; Coschigano and Wensink, 1993; An and Wensink, 1995); however, they mediate opposite effects on transcription, with Dsx<sup>M</sup> repressing transcription, and Dsx<sup>F</sup> promoting transcription (Garabedian *et al.*, 1986; Burtis *et al.*, 1991; Coschigano and Wensink, 1993). Thus, the opposite effects of the binding of Dsx's sex-specific isoforms on *yolk protein* transcription represent one mechanism by which a single gene, like *dsx*, can control the development of two different sexes.

### 1.2.2.4 *dsx* in the CNS

Although *dsx* is responsible for controlling the differentiation of primary sexual characteristics (pigmentation, reproductive organs, external genitalia), it also plays a role in the development of a sexually dimorphic CNS. Taylor and Truman (1992) showed that Dsx<sup>M</sup> was required to prolong the number of neuroblast divisions in the abdominal ganglion (Abg) in males, resulting in more neurons in the male Abg. More recently, Dsx has been shown to be expressed in the CNS, in approximately 600 neurons in males (Lee *et al.*, 2002). Thus *dsx* has a critical role in determining the sex of non-neuronal tissues, but is also involved in aspects of sex-specific development of the CNS.

### 1.2.2.5 *dsx* is Required for Male Sexual Behaviour

In addition to its role in determining external and internal sexual characteristics and biochemistry, *dsx* is also required for wild-type courtship behaviour (McRobert and Tompkins, 1985; Taylor *et al.*, 1994; Villella and Hall, 1996). *dsx* mutant males perform less courtship behaviour compared to wild-type males, and also spend significantly less time performing specific steps of courtship such as wing extension and licking (McRobert and Tompkins, 1985; Taylor *et al.*, 1994; Villella and Hall, 1996). Recent reports have suggested that at least some of these courtship defects are due to the loss of expression of a taste receptor

gene called *Gr68a* in *dsx* mutants (Bray and Amrein, 2003). *Gr68a* is a gustatory receptor gene expressed in a *dsx*-dependent manner on the male-specific taste bristles on the foreleg, and is required for wild-type courtship (Bray and Amrein, 2003). Thus one way in which the effects of *dsx* on courtship behaviour may be mediated is by controlling the expression of genes involved in mate recognition. A further behavioural phenotype of *dsx* mutant males is a defect in one of the two components of courtship song, called sine song, where *dsx* mutant males do not perform sine song (Villella and Hall, 1996); however, the neurobiological aetiology of the aberrant song phenotype has not yet been determined.

Thus *dsx* is involved in the specification of some aspects of behaviour, but can *dsx* expression alone specify male-specific behaviours? Using the *dsx*<sup>Dom</sup> allele of *dsx*, in which only the male-specific isoform of *dsx* is constitutively expressed in heterozygous individuals (over a *dsx* deficiency), Taylor *et al.* (1994) showed that the expression of *Dsx*<sup>M</sup> in females could not trigger male courtship. Thus, while *dsx* controls internal and external morphology, as well as a sexually dimorphic pheromonal profile, it seems that it is not sufficient to specify male sexual behaviour, suggesting that another gene(s) is largely responsible for the specification of male sexual behaviour.

### **1.2.3 fruitless Controls Male Sexual Behaviour**

A mutation in *fru* (*fru*<sup>1</sup>) was originally isolated in a screen for male sterility (Gill, 1963), where the sterility was later linked to abnormalities in courtship behaviour and a failure to attempt copulation (Hall, 1978; Gailey and Hall, 1989). Further *fru* mutant alleles (*fru*<sup>3</sup>, *fru*<sup>4</sup>, *fru*<sup>sat</sup>) were subsequently identified in screens for male sterility (Castrillon *et al.*, 1993; Ito *et al.*, 1996), and also through gene targeting by homologous recombination (*fru*<sup>F</sup>) (Demir and Dickson, 2005). None of these mutations in *fru* have any discernible effects on the sexual behaviour of females (Hall, 1978; Ito *et al.*, 1996; Villella *et al.*, 1997; Demir and Dickson, 2005), and, in contrast to *dsx*, do not affect the development of external or internal sexual characteristics; findings which have led to the suggestion that *fru* exclusively controls male sexual behaviour in *Drosophila* (Taylor *et al.*, 1994; Baker *et al.*, 2001; Demir and Dickson, 2005).

### 1.2.3.1 Transcripts from *fru*'s P1 Promoter Are Sex-Specifically Spliced and Expressed

The *fru* locus spans approximately 150 kb, from cytogenetic location 91A7-91B3, and is thought to encode a BTB (Broad-complex/Tramtrack/Bric-à-brac)-Zf transcription factor, with C2H2-type zinc fingers (Ryner *et al.*, 1996; Ito *et al.*, 1996). *fru* is a transcriptionally complex gene, with alternative splicing at both the 5' and 3' ends (Figure 1.4) (Ito *et al.*, 1996; Ryner *et al.*, 1996). *fru* transcripts are generated from one of four promoters (P1-P4), where transcripts originating from the P2-P4 promoters are non-sex-specific, and perform many of *fru*'s vital functions (Ryner *et al.*, 1996; Goodwin *et al.*, 2000; Anand *et al.*, 2001). Transcripts from the P1 promoter, on the other hand, are sex-specifically spliced at the 5' end by the female-specific splice factor Tra and non-sex-specific Tra-2 (Ryner *et al.*, 1996; Heinrichs *et al.*, 1998; Goodwin *et al.*, 2000). In females, pre-mRNA from the P1 promoter undergoes sex-specific splicing mediated by Tra and Tra-2, introducing a stop codon into the S exon; as a result, no functional Fru protein is made from these P1 transcripts (Lee *et al.*, 2000). In males, however, in the absence of Tra and Tra-2, a default splice site is used, and a functional Fru protein is made from these P1 transcripts, resulting in a class of male-specific Fru proteins collectively referred to as Fru<sup>M</sup> proteins (Lee *et al.*, 2000). It is these male-specific Fru<sup>M</sup> proteins that are thought to mediate *fru*'s critical role in the specification of male sexual behaviour (Ryner *et al.*, 1996; Villella *et al.*, 1997; Goodwin *et al.*, 2000; Demir and Dickson, 2005).

In addition to this Tra/Tra-2 mediated sex-specific splicing at the 5' end of P1 transcripts, pre-mRNAs from all promoters are subject to alternative splicing at the 3' end, where each transcript will contain a BTB protein-protein interaction domain, and in theory, one of four alternative 3' zinc finger ends, A, B, C or D (nomenclature as per Billeter *et al.*, 2006b; Ryner *et al.*, 1996; Usui-Aoki *et al.*, 2000). It has been demonstrated, however, that the male-specific *fru* transcripts originating from P1 only contain zinc fingers A, B or C in the CNS (Billeter *et al.*, 2006b).

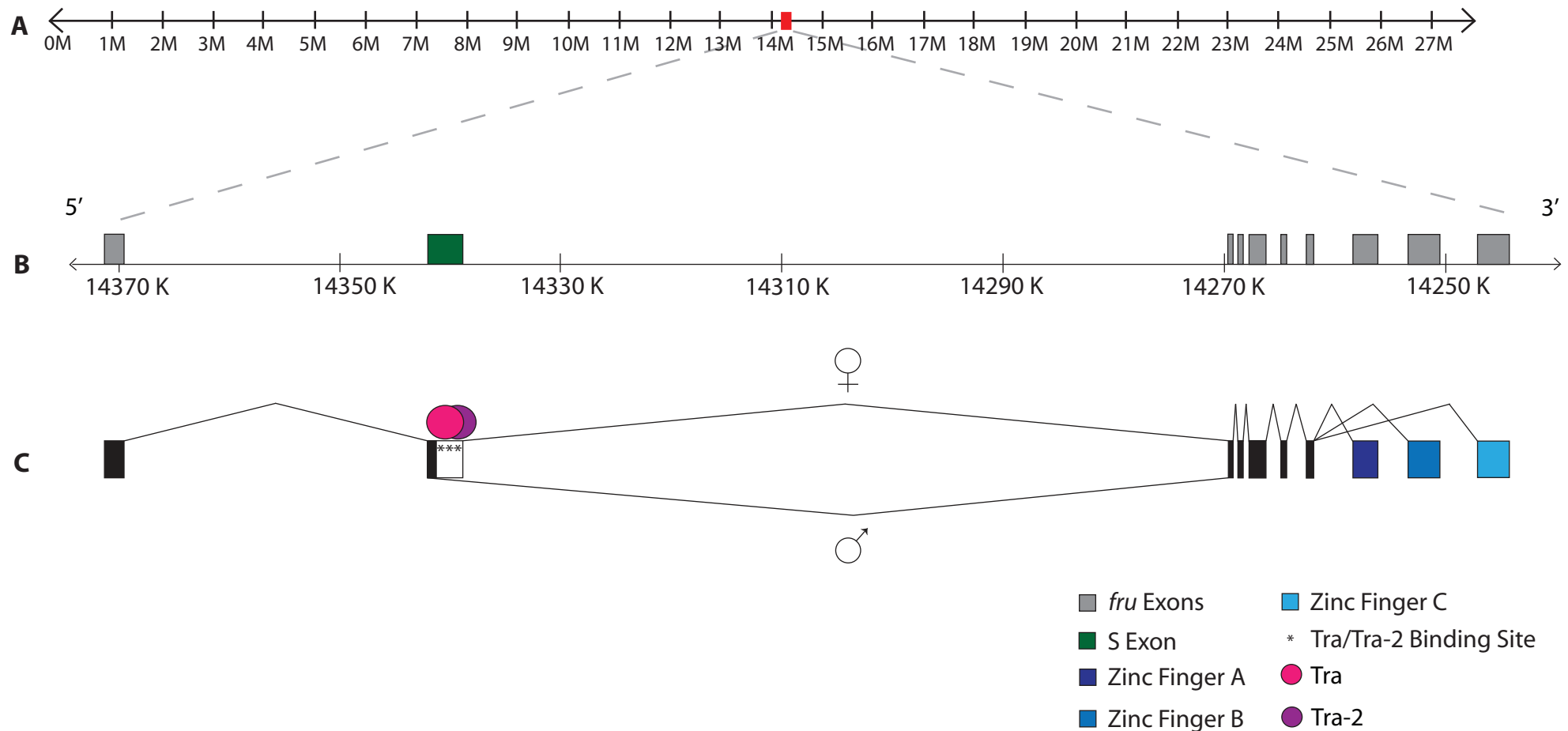
### 1.2.3.2 Fru<sup>M</sup> in the CNS

Although *fru* has both sex-specific and vital functions, only the sex-specific isoforms of *fru* will be addressed in this thesis, as only these male-specific Fru<sup>M</sup>

proteins are implicated in the determination of male sexual behaviour (Ryner *et al.*, 1996; Villella *et al.*, 1997; Goodwin *et al.*, 2000; Demir and Dickson, 2005).

Fru<sup>M</sup> expression is restricted to the CNS and PNS, where it is expressed in approximately 2000 neurons (Ryner *et al.*, 1996; Lee *et al.*, 2000; Billeter and Goodwin, 2004; Stockinger *et al.*, 2005; Billeter *et al.*, 2006b). Many regions in which Fru<sup>M</sup> is expressed are associated with male-specific behaviours (Hall, 1977; von Schilcher and Hall, 1979; Ferveur and Greenspan, 1998; Lee *et al.*, 2000; reviewed by Villella and Hall, 2007). For example, Fru<sup>M</sup> is expressed in the Abg of the CNS, a region which has been implicated by sex mosaic studies to be critical for attempted copulation and copulation (Hall, 1977; Hall, 1979; Ferveur and Greenspan, 1998; Lee *et al.*, 2000; Billeter *et al.*, 2006b). Likewise, Fru<sup>M</sup> is also expressed in regions of the posterior brain associated with behaviours such as licking, tapping and wing extension (Hall, 1977; Ferveur and Greenspan, 1998; Lee *et al.*, 2000). Thus the expression pattern of Fru<sup>M</sup> suggests a role in the creation of a sexually dimorphic CNS, capable of performing male behaviours.

Given that Fru<sup>M</sup> is expressed in regions associated with courtship behaviours, a number of studies have investigated the mechanism by which Fru<sup>M</sup> expression specifies the sexually dimorphic differentiation of the CNS. One way in which Fru<sup>M</sup> expression has been shown to create these differences is by directing the male-specific expression of serotonin in a cluster of neurons in the Abg (Lee and Hall, 2001). These neurons innervate the male internal reproductive organs, and the lack of serotonin in these neurons is associated with infertility (Lee and Hall, 2001; Lee *et al.*, 2001; Billeter and Goodwin, 2004). Thus Fru<sup>M</sup> might control aspects of male behaviour by specifying the neurotransmitter that is expressed in a group of neurons in the CNS.



**Figure 1.4-** Schematic Representation of the Transcripts Originating from *fru*'s P1 Promoter. (A) Right arm of the third chromosome is shown, with the *fru* locus indicated by a red box. M = megabases. (B) The *fru* locus spans approximately 150 kb on the right arm of the third chromosome, from cytogenetic location 91A7-91B3. (C) Transcripts from the P1 promoter undergo sex-specific splicing at the 5' end of the transcript mediated by the female-specific splice factor Tra in conjunction with non-sex-specific splice factor Tra-2. In the CNS, P1 transcripts from both sexes are alternatively spliced at the 3' end to incorporate one of three zinc fingers ends (A,B or C) into the transcript.

Another way in which Fru<sup>M</sup> has been shown to create differences between the male and female CNS is by specifying the development and morphology of a group of neurons in the brain above the antennal lobe. These neurons are Fru<sup>M</sup>-expressing (in males), and the number of neurons in the cluster is sexually dimorphic (Kimura *et al.*, 2005). Kimura *et al.* (2005) showed that this dimorphism was a direct result of Fru<sup>M</sup> expression, where Fru<sup>M</sup> expression prevented programmed cell death exclusively in males. In addition, the projections of these neurons were shown to be sexually dimorphic, and again, Fru<sup>M</sup> expression was responsible for creating the sex-specific morphology (Kimura *et al.*, 2005). Thus Fru<sup>M</sup> plays a critical role in the differentiation of a male-specific CNS by exploiting a number of developmental and physiological pathways.

### 1.2.3.3 *fru* Is Necessary for Male Sexual Behaviour

Mutations affecting the expression of Fru<sup>M</sup> proteins have been shown to control most aspects of male sexual behaviour in *Drosophila*. Certain mutant combinations of *fru* display minimal amounts of courtship behaviour, and are able to mate (Ryner *et al.*, 1996; Villella *et al.*, 1997; Goodwin *et al.*, 2000); however, in spite of having motile sperm, these mutants have difficulty in transferring sperm and seminal fluids, and have extremely lengthy copulations (Lee *et al.*, 2001). Other *fru* mutant allelic combinations perform little or no courtship, and never copulate (Hall, 1978; Gailey and Hall, 1989; Ryner *et al.*, 1996; Villella *et al.*, 1997; Goodwin *et al.*, 2000). Males with allelic combinations causing the most aberrant courtship behaviour have also been shown to have defects in one of the two components of courtship song, called pulse song, where *fru* mutant males perform no pulse song (Ryner *et al.*, 1996; Villella *et al.*, 1997; Goodwin *et al.*, 2000). Together, these results demonstrate that Fru<sup>M</sup> is required for the specification of male sexual behaviour.

Normally, females do not express Fru<sup>M</sup> proteins, and appear to be totally unaffected by mutations which disrupt the expression of Fru<sup>M</sup> (Ito *et al.*, 1996; Villella *et al.*, 1997). However, to investigate whether Fru<sup>M</sup> expression alone was responsible for the specification of behaviour, in the absence of any other genetic factors, novel alleles of *fru* were made, called *fru*<sup>M</sup> and *fru*<sup>Δtra</sup>, in which *fru* transcripts are constitutively spliced in a male fashion in both sexes (Demir

and Dickson, 2005). This splicing is achieved either by the deletion of the entire female-specific portion of the S exon, which includes the Tra/Tra-2 binding sites in *fru*<sup>M</sup> females; or the deletion of only the Tra/Tra-2 binding sites from the S exon in *fru*<sup>Δtra</sup> females (refer to Figure 1.4). Although Tra/Tra-2 are both present in these females, the sex-specific splice of the *fru* transcript is not possible without the binding sites, resulting in the use of the default male splice site, and the production of Fru<sup>M</sup> proteins in females.

When the courtship behaviour of *fru*<sup>M</sup> and *fru*<sup>Δtra</sup> females was examined, these females were found to perform some aspects of the courtship ritual, albeit at subnormal levels (Demir and Dickson, 2005). *fru*<sup>M</sup> and *fru*<sup>Δtra</sup> females were shown to orient, follow, tap, extend their wings, and lick; however, they never attempted copulation, and it was not determined whether they produced any courtship song. Therefore, the expression of Fru<sup>M</sup> in females can specify some, but not all male behaviours, suggesting that while Fru<sup>M</sup> is a critical regulator of male sexual behaviour, it is not sufficient to specify all male behaviours.

#### **1.2.4 Do *fru* and *dsx* Interact?**

Given that *tra* mutant females, who express the male-specific isoforms of *fru* and *dsx*, are able to perform all male-specific behaviours (Kyriacou and Hall, 1980; Bernstein *et al.*, 1992), *tra* must be able to regulate all downstream events required for courtship behaviour. Typically, *dsx* and *fru* are thought to sit atop independent branches of the sex determination pathway, where *dsx* governs the development of primary sexual characteristics, and *fru* is responsible for specifying the sexually dimorphic development of the CNS and male-specific behaviours (reviewed by Billeter *et al.*, 2002; Billeter *et al.*, 2006a). Indeed, the expression of Fru<sup>M</sup> in females can induce the performance of many male behaviours, however, these Fru<sup>M</sup>-expressing females perform subnormal amounts of courtship, do not attempt copulation, and it is not known whether they are capable of performing courtship song. Thus Fru<sup>M</sup> expression alone is not solely responsible for the specification of a developmental and/or physiological program of events underlying courtship behaviour. Instead, it seems likely that other gene(s) are required to act alongside Fru<sup>M</sup> in the specification of male behaviours. Although *dsx* alone cannot specify courtship behaviour, it is required for specific behaviours such as wing extension,

courtship song, licking and tapping (McRobert and Tompkins, 1985; Taylor *et al.*, 1994; Villella and Hall, 1996). In addition, both isoforms of *Dsx* are expressed in the CNS, and *Dsx<sup>M</sup>* been shown to play a role in the creation of a sexually dimorphic CNS (Lee *et al.*, 2002; Taylor and Truman, 1992). Thus *dsx* is the obvious candidate to act alongside *Fru<sup>M</sup>* in the specification of some aspects of courtship behaviour.

#### 1.2.4.1 Courtship Song

Many studies have documented how mutations in either *fru* or *dsx* affect the performance of courtship behaviour and the sexually dimorphic development of the CNS, yet few have examined both the individual and combined contributions of *fru* and *dsx* to the specification of a single component of the courtship ritual. Therefore, in this thesis, the individual and/or combined requirement for *fru* and *dsx* in the production of courtship song will be investigated; in addition, the relative contribution of each gene to the creation of a sexually dimorphic CNS capable of song production will be ascertained. As such, the remaining introduction will discuss the importance of courtship song in *Drosophila*, what is known about the neurobiology of courtship song, and the roles of *fru* and *dsx* in the specification of courtship song.

### 1.3 Acoustic Communication in *Drosophila*

Many animals use acoustic communication to locate and attract a mate (reviewed by White and Barfield, 1990; Tauber and Eberl, 2003; Kelley, 2004; Hedwig, 2006). In *Drosophila*, the ‘lovesong’ produced by the male during the courtship ritual both promotes mating, and communicates vital species-specific information (Bennet-Clark and Ewing, 1969; Kyriacou and Hall, 1980; Kyriacou and Hall, 1982; Kyriacou and Hall, 1984; Kyriacou and Hall, 1986; Rybak *et al.*, 2002; reviewed by Gleason, 2005). In addition, courtship song likely contributes to sexual isolation between species of the *melanogaster* group (Bennet-Clark and Ewing, 1967; Bennet-Clark and Ewing, 1969; Kyriacou and Hall, 1986; Ritchie *et al.*, 1999; Tomaru *et al.*, 2000; Rybak *et al.*, 2002). Thus by investigating the genetic and neurobiological basis for courtship song, a better understanding of how sexually dimorphic behaviours such as song are programmed into the CNS



can be gained; along with a greater understanding of the types of genetic changes underlying reproductive isolation (Gleason *et al.*, 2002).

#### **1.3.1.1 Recording Song in *Drosophila***

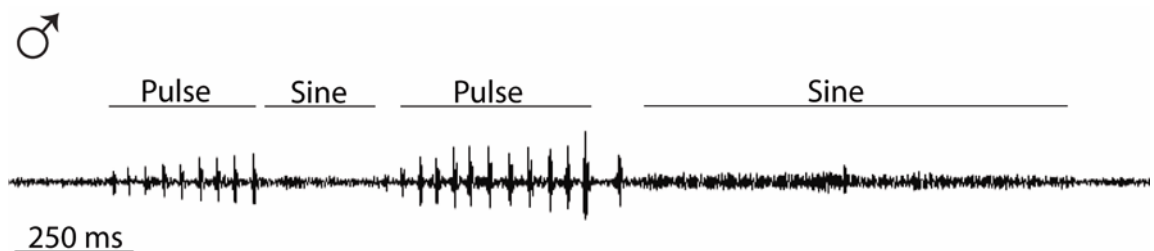
Sound has two components: a pressure component, and a particle displacement component, where the particle displacement component of sound decays sharply over distance, making it suitable for communication only at short distances (reviewed by Tauber and Eberl, 2003). The vibrations of the male's wing in close proximity to the female result in a particle velocity of approximately 80-90 dB (Bennet-Clark, 1971); however, as a result of the directional output of the song and the decay in particle velocity over distance, few individuals other than the intended target are able to detect the song. This presents a significant problem for researchers who would like to record and analyze courtship song in *Drosophila*. Thus, specialized song recording equipment has been developed to allow researchers to better record and analyze the courtship song (Gorczyca and Hall, 1987).

Flies 'hear' by detecting the particle displacement component of sound with their antennae. A specialized hair on the antenna called the arista detects the air displacement (Manning, 1967b), and its vibration causes the rotation of the third antennal segment, which then activates mechanoreceptors on the second antennal segment (Göpfert and Robert, 2002); however, mechanistic details of this mechano-electrical transduction are not known (reviewed by Tauber and Eberl, 2003). In order for researchers to 'hear', record and analyze song, a specific type of microphone able to detect the particle displacement component of sound, called a particle velocity microphone, is employed to detect the courtship song. Gorczyca and Hall (1987) devised an integrated recording device called the INSECTAVOX, which employs a particle velocity microphone coupled to amplifiers and a power source, in which all three components are contained in separate, insulated chambers to minimize environmental and electronic noises on the recording.

#### **1.3.2 The Courtship Song of *Drosophila melanogaster***

Courtship song in *Drosophila melanogaster* is a male-specific behaviour, and consists of a low frequency humming sound called a sine song (von Schilcher,

1976c), and a rhythmically patterned pulse song (Figure 1.5) (Ewing and Bennet-Clark, 1968). Sine song has a carrier frequency of approximately 160 Hz, and the sound is produced by beating the wing in a manner similar to that seen in flight (von Schilcher, 1976c). Pulse song, on the other hand, consists of a series of monocyclic pulses, and has an intrapulse carrier frequency of approximately 240 Hz (Ewing and Bennet-Clark, 1968; Wheeler *et al.*, 1988). Two parameters of pulse song have been shown to communicate vital species-specific information: the length of the time between consecutive pulses, known as the interpulse interval (IPI), and the sinusoidal cycling of the IPI (Bennet-Clark and Ewing, 1967; Bennet-Clark and Ewing, 1968; Kyriacou and Hall, 1980; Kyriacou and Hall, 1982; Kyriacou and Hall, 1986).



**Figure 1.5**-Representative Song Trace of *Drosophila melanogaster*. Courtship song consists of a rhythmically patterned pulse song and a low frequency humming sound called sine song, both of which are indicated above the trace.

In *Drosophila melanogaster* the mean length of the IPI is approximately 33 ms (Shorey, 1962; Bennet-Clark and Ewing, 1967; Bennet-Clark and Ewing, 1968; Kawanishi and Watanabe, 1980), whereas in other species, such as *D. simulans*, the IPI is approximately 50 ms (Ewing and Bennet-Clark, 1968; Kawanishi and Watanabe, 1980). In addition to the length of the IPI, the sinusoidal cycling of the IPI, which has a period of approximately 55-60 s in *Drosophila melanogaster*, has also been shown to be species-specific (Kyriacou and Hall, 1980; Kyriacou and Hall, 1982). These species-specific parameters of courtship song are critical in the identification of conspecific males by females, thus it is not surprising that several studies have shown that females greatly prefer courtship song with the IPI and IPI cycling of their own species to either no song, or song with the incorrect species parameters (Kyriacou and Hall, 1982; Ritchie *et al.*, 1999).

Clearly, courtship song is a critical component of the courtship ritual in *Drosophila melanogaster*; however, the mechanism by which the sexually dimorphic nature of song production is achieved remains unknown.

### 1.3.2.1 Genetics of Sex-Specific Song Production in *Drosophila*

A number of genes affecting song production have been identified, including *period* (*per*), *cacophony* (*cac*), *no-on-transient A* (*nonA*), *tra*, *dsx* and *fru*, amongst others (Kyriacou and Hall, 1980; von Schilcher, 1976b; von Schilcher, 1977; Kulkarni *et al.*, 1988; Stanewsky *et al.*, 1996; Bernstein *et al.*, 1992; Villella and Hall, 1996; Ryner *et al.*, 1996; Villella *et al.*, 1997; Goodwin *et al.*, 2000). Of these genes, however, only *tra*, *dsx* and *fru* are involved in the specification of sexual identity and morphology. Therefore, an investigation into how genes direct the sex-specific development of the CNS in order to allow song production must focus on the individual and combined contributions of these sex determination genes.

Mutations in *tra* do not have any discernible effects on the courtship behaviour of males (McRobert and Tompkins, 1985); however, females lacking *Tra* are transformed into pseudomales, and are able to display all male behaviours, including courtship song (Brown and King, 1961; McRobert and Tompkins, 1985; Kyriacou and Hall, 1980; Bernstein *et al.*, 1992). Therefore, *tra* must regulate all downstream events required for the specification of external and internal sexual characteristics, and the sex-specific development of the CNS, to permit courtship song production. Two downstream targets of *tra*, *fru* and *dsx*, are also required for courtship song, suggesting that these genes are responsible for triggering the events required to generate the potential for courtship song in a given individual.

Mutations in both *dsx* and *fru* disrupt courtship song; *dsx* mutants fail to produce the sine song component of courtship song, whereas *fru* mutants fail to produce the pulse song component. Although *fru* and *dsx* are thought to govern independent branches of the sex determination pathway in *Drosophila*, the fact that mutations in either gene cause aberrant song production suggests that these genes may act in concert, or in parallel, to influence the sexually dimorphic development of structures required for song production. What then are these critical differences? And where might they lie?

### **1.3.2.2 Singing and the Brain**

Courtship song is generated when the male fly rapidly vibrates his wing, and must therefore involve the musculature allowing these precise wing movements. However, the ability to produce courtship song relies not upon the sex of the wing and the wing's musculature, but upon the sex of the CNS (Hotta and Benzer, 1976; Hall, 1977; Hall, 1979; von Schilcher and Hall, 1979; Greenspan and Ferveur, 1998). Studies using male-female mosaics have shown that the absence of male tissue in specific regions of the CNS results in aberrant or absent song, even when the wing and its associated muscles are male (von Schilcher and Hall, 1979). Furthermore, provided that male tissue in these specific regions of the CNS is present, female tissue in the wing and surrounding musculature does not preclude the production of courtship song (von Schilcher and Hall, 1979). Together, these results suggest that the anatomical focus of the sexually dimorphic production of courtship song lies in the CNS.

### **1.3.2.3 Song Foci in the Brain**

By testing the courtship behaviour and song production in male-female mosaics, specific regions of the CNS were implicated in the generation of courtship song (von Schilcher and Hall, 1979; Konopka *et al.*, 1996). Given that courtship song requires the initiation of courtship behaviour, it is not surprising that the regions of the brain implicated in the initiation of courtship behaviours, such as the dorsal posterior brain, needed to be male in order for song production to occur (Hall, 1979; von Schilcher and Hall, 1979). However, among the mosaics capable of initiating courtship behaviour, it was determined that the regions of the CNS which mapped closest to the neural foci for courtship song were the ventral thoracic ganglia of the CNS, more specifically, the mesothoracic ganglion (Msg) (von Schilcher and Hall, 1979; Konopka *et al.*, 1996). Thus it seems likely that the critical differences between the sexes underlying the sexually dimorphic production of courtship will be found in this region.

### **1.3.2.4 Neurobiology of Courtship Song**

Although no dimorphisms in the Msg have been reported, sex-specific developmental and physiological processes have been reported in other regions

of the CNS, suggesting that many factors influence the sexually dimorphic production of courtship song.

For example, Taylor and Truman (1992) showed that a male-specific population of neurons was created by sexually dimorphic neurogenesis in the Abg. Alternatively, sexually dimorphic neuronal populations can be generated by sex-specific programmed cell death (Kimura *et al.*, 2005). In a cluster of neurons just above the antennal lobe, Kimura *et al.* (2005) showed that adult males have more neurons than females; where this dimorphism was created by sex-specific programmed cell death in females. Thus sex-specific neurogenesis and programmed cell death are two mechanisms by which a sexually dimorphic CNS can be created, where these sex-specific populations of neurons can then be arranged or incorporated into new or existing neural circuits, ultimately leading to the performance of sexually dimorphic behaviours.

In addition to the creation of sexually dimorphic neuronal populations, a further mechanism by which a sexually dimorphic behavioural output can be achieved involves the development of sex-specific neuroanatomical or neurophysiological properties. For example, the neurons innervating the male internal reproductive organs are serotonergic (Lee and Hall, 2001), however, these neurons are either not present, or simply do not express serotonin in females, suggesting that sex-specific copulatory behaviours may be at least partly due to the sexually dimorphic expression of a neurotransmitter. In addition, Kimura *et al.* (2005) not only showed that a cluster of neurons located just above the antennal lobe was sexually dimorphic in the number of neurons, but also that the morphology of the neurons in this cluster was sex-specific. Although the significance of this dimorphic neuronal morphology is not yet known, further experiments will confirm the precise role of these morphological differences in creating sex-specific behaviours. One final example in which the sexually dimorphic function of the CNS has been demonstrated to lead to sexually dimorphic behaviour is in larval crawling behaviour. Larval locomotion is sexually dimorphic (Glossop and Shepherd, 1998), and Lnenicka *et al.* (2006) propose that this dimorphism is a consequence of increased neurotransmitter release by female boutons. Thus even in the absence of any differences in neurotransmitter release or bouton morphology and/or size, sexually dimorphic behaviour can be achieved simply by dimorphic neurotransmitter release at the neuromuscular junction (NMJ).

Therefore, given the critical role of the sex of the *Msg* in the specification of courtship song, it seems likely that a detailed examination of the neurobiological properties of this region will reveal the developmental and/or physiological mechanisms which allow the sex-specific production of courtship song. Moreover, given the known roles of *dsx* and *fru* in creating dimorphisms both in the CNS and PNS, the respective roles of these genes in the creation of a sexually dimorphic *Msg* can be examined. Thus, in the next section, the techniques that will be employed to investigate the genetic and neurobiological basis for the sexually dimorphic production of courtship song will be discussed.

## **1.4 Genetic and Neurobiological Dissection of Behaviour in *Drosophila***

In order to understand the connection between a gene and a particular behavioural outcome, it must be possible to determine how, where and when a gene's product acts during development to specify some aspect of neuronal development or function. Next, by manipulating the function and/or presence of individual neurons, the relationships between individual neurons involved in a specific behaviour can be discovered, and a neural circuit underlying this behaviour can be identified. Given that *Drosophila* has been used as a model organism for the investigation of the genetic basis for behaviour for many years, numerous techniques have been developed for the generation of new mutants, the ectopic expression of genes, and the activation or disruption of neuronal function (Brand and Perrimon, 1993; Rong and Golic, 2000; Kitamoto, 2001; Lima and Miesenböck, 2005; Venken and Bellen, 2007).

### **1.4.1 Genetic Dissection of Behaviour**

Understanding the genetic basis for behaviour demands that it be possible to examine whether a given gene is required for a behaviour by making mutations in that gene, and also to express that gene in a spatially and temporally restricted manner, to determine whether that gene alone can dictate the developmental and/or functional events necessary for the performance of that behaviour. In *Drosophila*, mutations or deletions in many genes have been reported, and in addition, a system for targeting mutations to genes of unknown function has been developed, allowing the behavioural requirements for single

genes to be determined. Moreover, the UAS/GAL4 binary transcription system has long been used to express genes of interest in a temporally and spatially restricted/controlled manner, allowing the investigation of gene function in small groups of cells and/or neurons (reviewed by Duffy, 2002; Venken and Bellen, 2007).

#### 1.4.1.1 Homologous Recombination

Gene targeting by homologous recombination is a relatively new technique in *Drosophila*, first described by Rong and Golic (2000). It is based on the well-established technique of *P*-element transformation (Rubin and Spradling, 1983), and a number of loci have been successfully targeted for mutation and/or modification using homologous recombination (Rong and Golic, 2000; Rong and Golic, 2001; Rong *et al.*, 2002; Manoli *et al.*, 2005; Demir and Dickson, 2005; Stockinger *et al.*, 2005). Mutations in a gene of interest can be successfully isolated using *P*-element- or EMS (ethane methyl sulphonate)-mediated mutagenesis; however, one advantage of homologous recombination is the ability to make targeted insertions, deletions or specific alterations in a gene of interest. This is particularly important when manipulating very large genes with multiple promoters and/or protein isoforms, and genes with complex *cis*-acting regulation. Thus in some cases, despite being relatively labour intensive, gene targeting by homologous recombination is ideal for creating mutations to disrupt specific aspects of a gene's function (reviewed by Bi and Rong, 2003).

#### General Methodology and Genetic Crosses

Gene targeting by homologous recombination begins with the generation of a 'donor' *P*-element construct containing regions of homology to the targeted gene. Previous studies have used anywhere from 2.9 kb to 8.9 kb of donor:target homology (Rong and Golic, 2000; Rong and Golic, 2001; Rong *et al.*, 2002), though typically around 4 kb of donor:target homology is sufficient. The donor *P*-element will always contain *P*-element transposition sequences and FRT sites at each of the extreme 5' and 3' ends, flanking the regions of homology and other vector sequences. In addition to the donor:target homology, a marker gene, and a recognition site for the site-specific endonuclease I-*Scel* are all contained within the donor *P*-element. Once the donor *P*-element has been constructed, it is randomly inserted into the genome by *P*-element transformation technology

(Rubin and Spradling, 1983), and the process of gene targeting by homologous recombination can begin. Depending on the desired end product, the construct design is tailored to follow one of two alternative strategies of gene targeting by homologous recombination: ends-in or ends-out; both of which are described in greater detail below (Rong and Golic, 2000).

## Ends-Out

The ends-out strategy of gene targeting is most often used if the desired end product of the gene targeting by homologous recombination is simply a null mutation in the gene of interest (Figure 1.6) (Rong and Golic, 2000; Rong *et al.*, 2002). In addition, ends-out homologous recombination can be exploited to produce two different types of null mutations, based simply on the genomic region chosen as the donor:target homology (Rong and Golic, 2000; Rong *et al.*, 2002). If the donor:target homology consists of sequences within the targeted locus, the gene will be disrupted by the insertion of the entire donor construct. On the other hand, if the donor:target homology consists of sequences flanking the desired locus, then the entire gene is replaced by the donor construct (reviewed by Bi and Rong, 2003).

For ends-out gene targeting, the donor construct is designed such that the regions of donor:target homology are separated by the marker gene, and the I-*Scel* recognition site is not in close proximity to the regions of homology or the marker gene (Figure 1.6). Once the donor construct has been inserted into the genome at random by *P*-element transformation, a stereotypical series of steps is followed to generate a null mutant in the gene of interest (Rong and Golic, 2000; Rong and Golic, 2001; Rong *et al.*, 2002). First, the strain of flies carrying the donor *P*-element is crossed to a strain of flies carrying a site-specific recombinase (FLP) and site-specific endonuclease (I-*Scel*), both of which are heat-shock inducible. The progeny of this cross are heat-shocked early in development, which both excises the donor element from the genome, and induces a double-stranded break. This double-strand break-containing linearized donor element is then integrated at the desired locus by homologous recombination, where integration events are easily visible if a suitable genetic marker, such as *mini-white* (eye colour), is chosen. This integration event disrupts the gene to create the desired mutant, and in addition, has the



advantage of genetically marking the mutant (Figure 1.6). However, the presence of the marker in the genome can also be a disadvantage in some cases, as it may interfere with the phenotype under observation. For example, the presence of *mini-white* in the genome has been shown to trigger inappropriately high levels of male-male courtship (Zhang and Odenwald, 1995); therefore, if a mutation is made in a gene where the courtship of the newly-generated mutant will be analyzed, a second step must be incorporated into the ends-out strategy, to remove the interfering marker (as described by Stockinger *et al.*, 2005).

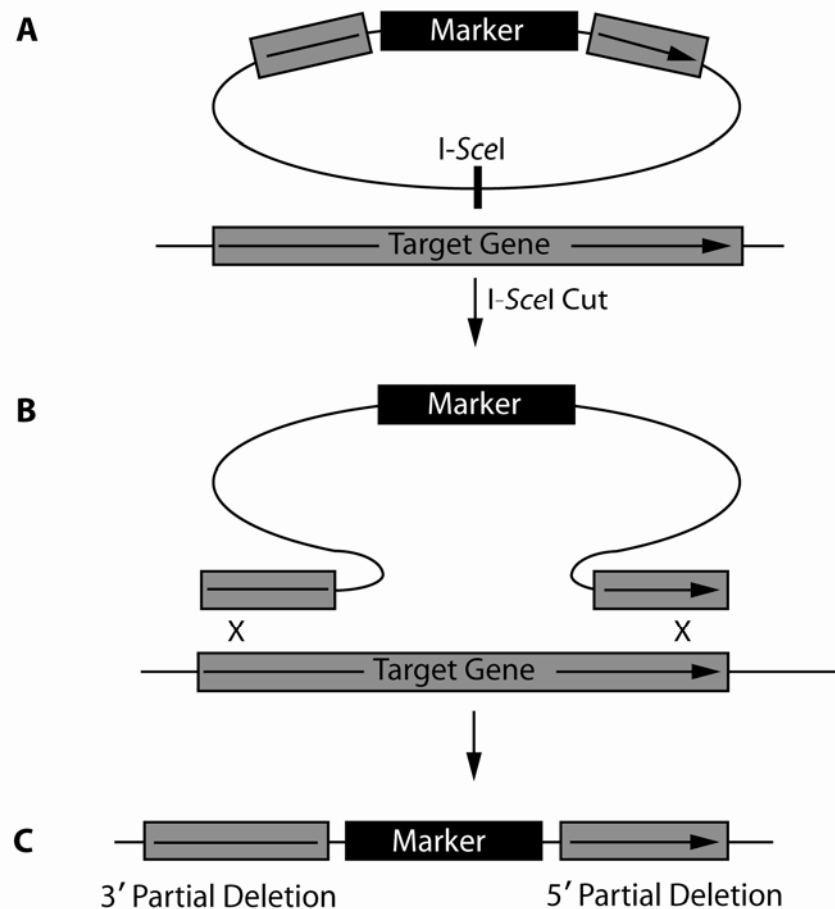
Although the ends-out strategy of gene targeting is ideal for creating null mutations in genes, the ends-in strategy of gene targeting is better suited to the creation of small deletions or insertions in a gene, or the disruption of a single isoform of a gene (reviewed by Bi and Rong, 2003).

### Ends-In

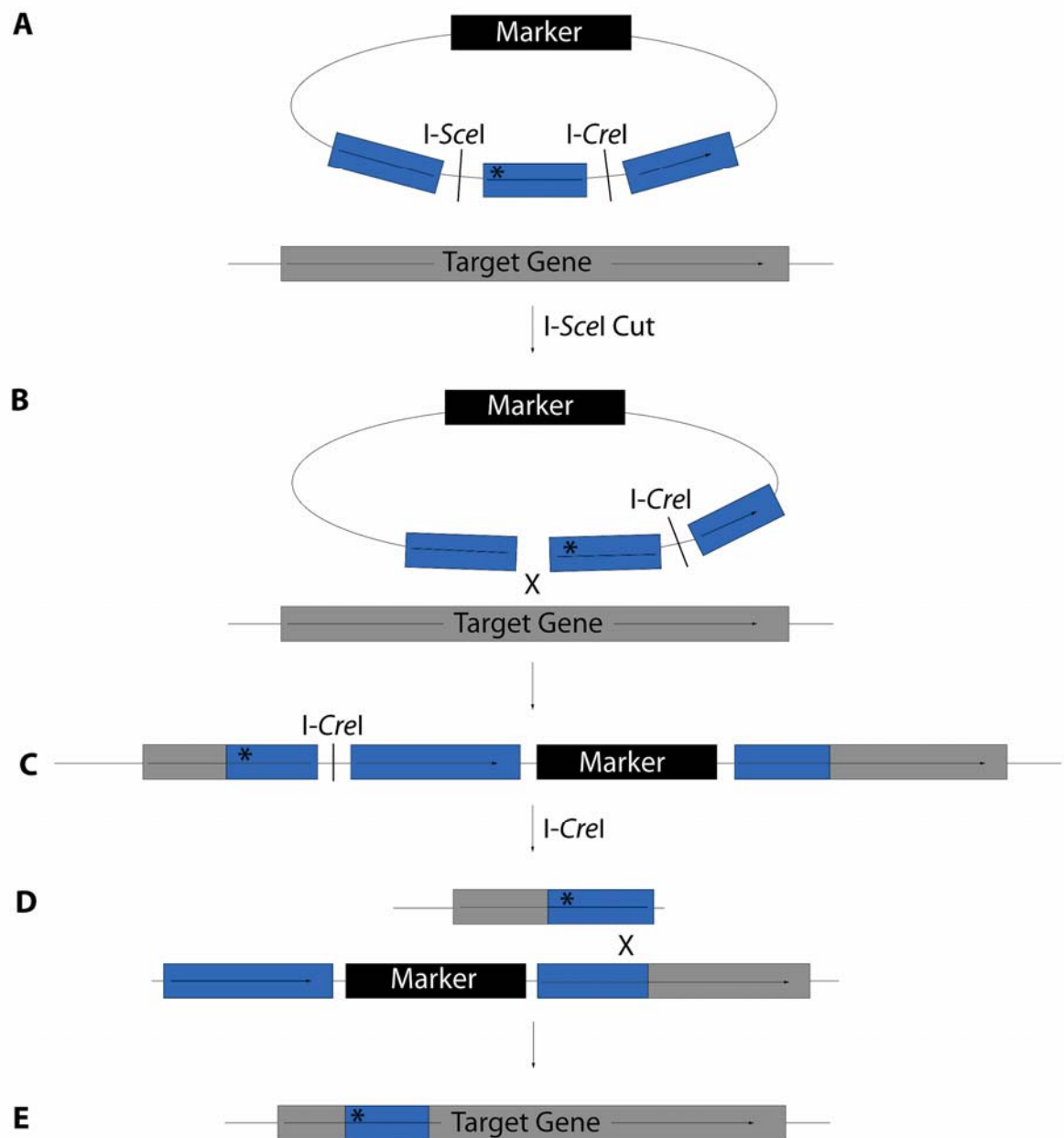
Generally, ends-in gene targeting is a two-step process; and although more time-consuming than the ends-out strategy, allows the generation of more subtle mutations in a gene of interest (Rong *et al.*, 2002). The donor construct for ends-in gene targeting is designed such that three regions of donor:target homology are used, and instead of being separated by the genetic marker, as in the ends-out donor construct, the three regions of homology are separated by recognition sites for the *I-SceI* and *I-CreI* site-specific endonucleases (Figure 1.7). Once the donor construct has been inserted into the genome at random by *P*-element transformation, two major steps are required to generate the desired alteration in the gene of interest. First, the strain of flies carrying the donor *P*-element is crossed to a strain of flies carrying a site-specific recombinase (FLP) and site-specific endonuclease (*I-SceI*), both of which are heat-shock inducible. The progeny of this cross are heat-shocked early in development, which both excises the donor element from the genome, and induces a double-strand break. This double-strand break-containing linearized donor element is then integrated at the desired locus by homologous recombination, where integration events are easily visible if a suitable genetic marker, such as *mini-white* (eye colour), is chosen. After the integration event, unlike ends-out gene targeting, the gene of interest is not replaced by the donor vector; instead, a duplication of the locus is created (Figure 1.7).

The second step of ends-in gene targeting is to resolve the duplication, which eliminates both the duplication and the marker gene. This is accomplished by crossing flies carrying the duplication to a strain of flies carrying a heat-shock inducible site-specific endonuclease called *I-Cre1*. The progeny of this cross are heat-shocked to induce a double-strand break in the locus, which is subsequently repaired by homologous recombination; ultimately resolving the duplication at the locus, and eliminating the marker gene (Figure 1.7) (Rong *et al.*, 2002). Although the two-step process of ends-in targeting is longer than the single-step ends-out gene targeting, small deletions or amino acid substitutions can be made in a gene; in addition, the marker is removed in the second step and does not interfere with the phenotype of interest.

Gene targeting by homologous recombination is an excellent tool by which a mutation in a gene of interest can be obtained, to allow the precise role of that gene in the specification of behaviour to be investigated.



**Figure 1.6**-Schematic Representation of Gene Targeting by Ends-out Homologous Recombination. (A) Targeting vector contains two regions of donor:target homology separated by a marker; the I-SceI recognition site is located at another location on the vector. (B) The donor vector is cut at the I-SceI recognition site, creating a double-stranded break. The donor vector is then integrated into the desired locus by homologous recombination. (C) Given that the regions of donor:target homology are located at either end of the targeted locus, integration of the vector at the targeted locus results in the deletion of any sequence located between the flanking regions of donor:target homology. Adapted from Rong and Golig (2000).



**Figure 1.7-Schematic Representation of Gene Targeting by Ends-in Homologous Recombination.** (A)The donor vector contains a genetic marker, and three regions of donor:target homology separated by the recognition sites for I-SceI and I-CreI restriction endonucleases. \* indicates the alteration to be made at the targeted locus. (B) A double-stranded break is made in the donor vector at the I-SceI site, and the vector is integrated into the target locus by homologous recombination. (C) A duplication is created at the targeted locus. (D) A double-stranded break is made at the I-CreI recognition site in the targeted locus, and the break is repaired by another round of homologous recombination. This repair results in the elimination of the marker gene, and the duplication in the targeted locus. (E) Resolved end product at the targeted locus, with only a single copy of the targeted gene, which now contains the desired alteration (\*). Adapted from Rong and Golio (2000).

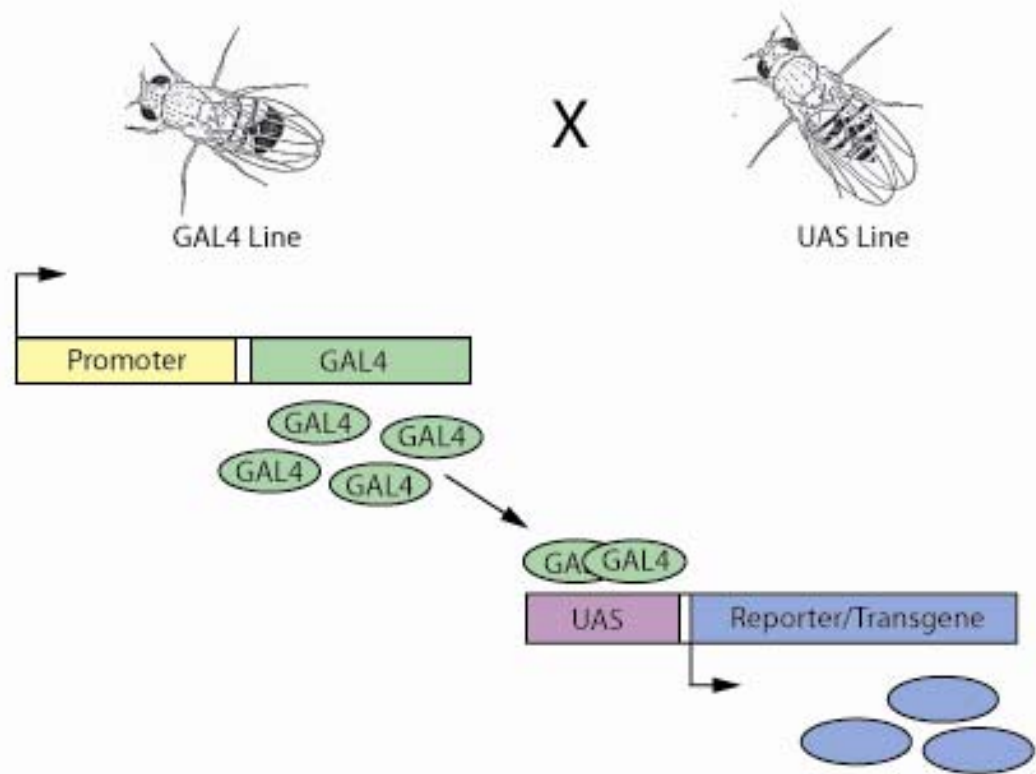
### 1.4.1.2 UAS/GAL4 System

After showing that a gene is involved in a given behaviour, investigators must then show where and when the gene is required to mediate these effects. In *Drosophila*, one of the most important genetic tools available to researchers is the UAS/GAL4 system of targeted gene expression (reviewed by Duffy, 2002).

GAL4 is a yeast protein which regulates transcription by binding directly to four related 17 bp sites, where these sites define an enhancer-like element called the upstream activating sequences (UAS) (Giniger *et al.*, 1985; Ptashne, 1988). GAL4 has no endogenous targets in *Drosophila*, and Fischer *et al.* (1988) showed that ectopic GAL4 expression could activate the transcription of reporter genes under the control of the UAS in *Drosophila*. This finding was extended by Brand and Perrimon (1993) to show that this UAS/GAL4 system of transcriptional activation could be used for targeted gene expression studies in *Drosophila* (Figure 1.8). Since these early reports on targeted gene expression, the UAS-GAL4 system has been widely used to investigate the genetic basis for both developmental processes and the specification of behaviour.

Virtually any gene in *Drosophila* can be either overexpressed, or ‘knocked down’ using the UAS/GAL4 system (reviewed by Duffy, 2002). Many GAL4 lines are available which drive expression at many different developmental times, in many different tissues, where these lines can be exploited to determine where and when a gene is either necessary and/or sufficient for a given behaviour. The UAS, on the other hand, can be fused to any sequence, allowing the expression of a desired gene to be manipulated. For example, by fusing the UAS to a transgene containing the entire coding sequence of a gene, the gene can be overexpressed in a desired tissue or at a specific developmental stage, and the consequences of this overexpression can be investigated (reviewed by Duffy, 2002). Conversely, fusing the UAS to a transgene containing inverted repeats of a gene would allow the gene to be ‘knocked down’ by the process of RNA interference in either specific tissues or developmental stages; again allowing the consequences of the decrease in expression to be assayed (reviewed by Duffy, 2002). Although a recent report suggests that the overexpression and subsequent accumulation of GAL4 can lead to neuronal cell death (Rezával *et al.*, 2007), if the appropriate controls for the action of GAL4 alone are

performed, the UAS/GAL4 system is one of the most versatile systems for investigating the genetic basis of behaviour.



**Figure 1.8**—The UAS/GAL4 System of Targeted Gene Expression in *Drosophila melanogaster*. Any promoter or promoter fragment can be fused upstream of GAL4, resulting in the expression of GAL4 in a spatial and temporal pattern as directed by the promoter in flies containing the promoter-GAL4 fusion. Similarly, any response elements, such as reporter genes or coding regions of a gene of interest, can be fused downstream of the UAS. When the flies containing the GAL4 and UAS transgenes are crossed, the promoter dictates the expression of GAL4 in a spatially and temporally restricted manner, which results in the expression of the reporter/gene of interest in GAL4-expressing cells. Adapted from Duffy (2002).

Together, gene targeting by homologous recombination and the UAS/GAL4 system are techniques which allow the investigation of how specific mutations in a gene (and its isoforms) control behaviour; and in addition, the investigation of the precise requirement for that gene in a given behaviour, by controlling gene expression in a temporally and spatially restricted manner.

### 1.4.2 Neurobiological Dissection of Behaviour

Understanding the neurobiological basis for behaviour requires the ability both to visualize and to manipulate neurons, so that specific neurons can be identified; and to disrupt neuronal function, to gain insight into the role of a

particular neurons in the generation of complex behaviours. In *Drosophila*, the UAS/GAL4 system has been exploited to allow researchers both to visualize and manipulate specific subsets of neurons, as described below.

#### 1.4.2.1 Neuroanatomy

One of the most important, and difficult, tasks in the investigation of the neurobiological basis for behaviour is to identify specific neurons, and to relate their anatomical and morphological characteristics to a particular function. In *Drosophila*, the UAS/GAL4 system is a powerful tool to identify stereotypical morphological and anatomical features of specific clusters of neurons. As discussed above, the UAS/GAL4 system can be used to identify regions of the CNS in which gene expression is required for a specific behaviour. Once GAL4 lines have been identified with expression in this region, the axonal and dendritic projections of specific subsets of neurons in this region can be examined using a UAS fused to the membrane-bound mouse lymphocyte marker CD8 (mCD8, which can also be conjugated to GFP) (Lee and Luo, 1999). These morphological characteristics can then be examined during development, and comparisons can be made either between mutant and wild-type strains, or between the sexes. Any differences can then be investigated with respect to function by simply examining where the neurons project, or by making single-neuron marked mutants using the MARCM system (mosaic analysis with a repressible cell marker) (Lee and Luo, 1999).

In flies, the antennal lobe (AL) is a region of the brain where the olfactory inputs converge, and from which the information is relayed to higher olfactory processing centres such as the mushroom bodies (MBs) (Vosshall and Stocker, 2007). Recently, studies examining the projections of olfactory receptor neurons (ORNs) showed that the axonal projections of sensory neurons expressing the same odorant receptor genes (*Or* genes) all projected to the same glomerulus in the AL (Couto *et al.*, 2005; Fishilevich and Vosshall, 2005; reviewed by Vosshall and Stocker, 2007). Thus function can at times be inferred by examining where the axons of a specific neurons project. Another example of inferring function from neuroanatomy is the projections of a cluster of serotonergic neurons in the Abg of male flies. These neurons were shown to innervate the male internal reproductive organs, and thus, were inferred to play an important role in

copulatory behaviours (Lee and Hall, 2001; Lee *et al.*, 2001; Billeter *et al.*, 2006b). Therefore, characterizing specific neurons using the UAS/GAL4 system can allow the behavioural significance of neuroanatomical features to be predicted and subsequently tested.

## 1.5 Aims and Objectives

Findings from a number of studies formed the basis for the work presented in this thesis. Courtship song is a sexually dimorphic behaviour, and is an important component of the male's courtship ritual, as it both promotes mating and communicates species-specific information (Bennet-Clark and Ewing, 1967; Bennet-Clark and Ewing, 1968; Bennet-Clark and Ewing, 1969; Kyriacou and Hall, 1980; Kyriacou and Hall, 1982; Ritchie *et al.*, 1999; Rybak *et al.*, 2002; reviewed by Gleason, 2005). Studies using male-female mosaics showed that specific regions of the CNS must be male in order for the production of courtship song (von Schilcher and Hall, 1979); however, the neurobiological mechanism by which the sexually dimorphic production of courtship song is achieved remains unknown.

Two genes in the sex determination hierarchy, *fru* and *dsx*, are required for song production (Ryner *et al.*, 1996; Villella and Hall, 1996; Villella *et al.*, 1997; Goodwin *et al.*, 2000); however, their individual and combined contributions to the specification of courtship song are unknown. Therefore, in this thesis, the overall objective was to investigate the neurobiological basis for the sex-specific production of courtship song, and to determine the roles of sex determination genes *fru* and *dsx* in creating the sexually dimorphic structures responsible for the production of courtship song.

In order to achieve this objective, the specific aims of this work were as follows:

- Compare the neurobiological properties of the motor neurons responsible for the production of courtship song in both males and females.
- Determine whether Fru<sup>M</sup> and Dsx are co-expressed in the CNS.
- Construct a genetic tool capable of directing expression in all *dsx* cells, by making a *dsx* promoter-GAL4 fusion, or using homologous recombination



to insert GAL4 into the *dsx* locus, to investigate the precise role of *dsx* in song production.

Ideally, a better understanding of the genetic and neurobiological basis for the sexually dimorphic production of courtship song will be gained, and will contribute to the current knowledge of the mechanism by which genes program sexually dimorphic behaviours into the CNS.

## **2 Materials and Methods**

## 2.1 *Drosophila*

### 2.1.1 *Drosophila* Stocks

A description of all the *Drosophila* stocks used in this work can be found in Table 2.1. A full description of all mutations and balancer chromosomes used can be found in FlyBase (<http://www.flybase.co.uk>).

Strain/Genotype	Description	Reference
<b>Reporter Transgenes</b>		
$w^h; P\{w^{mc}=UAS-mCD8::GFP.L\}LL5; +$	Fusion protein between mouse lymphocyte marker CD8 and the Green Fluorescence Protein (GFP). Labels cell membranes.	Lee and Luo, 1999
$w^h; +; P\{w^{mc}=UAS-lacZ.NZ\}J312$	Nuclear $\beta$ -Galactosidase.	Hiromi and West, Unpub.
$w^{1118}; +; P\{w^{mc}=UAS-StingerII\}$	Stable insulated nuclear enhanced GFP.	Barolo <i>et al.</i> , 2000
<b>Sex Determination Mutants</b>		
$Dp(1;Y)B^S Y; +; Df(3R)dsx^{15}/TM6B, Tb, Hu, e$	Deletion covering the <i>dsx</i> locus, from cytogenetic location 84DE5-84E6.	Baker <i>et al.</i> , 1991; Taylor <i>et al.</i> , 1994; Ring and Martinez Arias 1993
$w^+; +; dsx^1/TM3, Ser$	Homozygous individuals are intersexual in appearance. Spontaneous loss of function mutation in <i>dsx</i> .	Hildreth, 1965
$In(3R)dsx^{23}, dsx^{23}, Ubx^{bx-1}, sr^1, e^S/TM3, Sb^1$	Homozygous individuals are intersexual in appearance.	Duncan and Kaufman, 1975; Baker <i>et al.</i> , 1991
$w^1; +; dsx^{Sw}/TM3, Ser$	Small deletion in female-specific splice acceptor site in <i>dsx</i> . Dominant mutation where females heterozygous with deficiency <i>dsx</i> <sup>A3</sup> develop as pseudomales.	Baker and Wolfner, 1988; Nagoshi and Baker, 1990
$w^1; +; Df(3R)dsx^{A3}/TM3, Ser$	<i>dsx</i> deficiency, causes females to develop as pseudomales when heterozygous with <i>dsx</i> <sup>Sw</sup> .	Baker <i>et al.</i> , 1991; Waterbury <i>et al.</i> , 2000
$w^a; +; tra^1/TM2$	Homozygous chromosomal females differentiate as males. Spontaneous deletion removing most of the <i>transformer</i> ( <i>tra</i> ) locus.	Sturtevant, 1944; Butler <i>et al.</i> , 1986
$Dp(1;Y)B^S Y; +; Df(3L)st-J7$	Chromosomal deletion covering the <i>tra</i> locus, from cytogenetic location 73A2-73B2.	Belote <i>et al.</i> , 1990; Cunniff <i>et al.</i> , 1997

Strain/Genotype	Description	Reference
<b>fruitless Alleles</b>		
<i>fru<sup>M</sup>/TM3,Sb,Ser</i>	<i>fru</i> P1 transcripts are constitutively spliced in the male form due to the deletion of the entire female-specific S exon which contains the Tra/Tra-2 binding site.	Demir and Dickson, 2005
<i>fru<sup>Atra</sup>/TM3,Sb,Ser</i>	<i>fru</i> P1 transcripts are constitutively spliced in the male form due to the deletion of only the Tra/Tra-2 binding site.	Demir and Dickson, 2005
<i>fru<sup>F</sup>/TM3,Sb,Ser</i>	<i>fru</i> transcripts are constitutively spliced in the female form due to a mutation in the male splice donor site.	Demir and Dickson, 2005
<i>fru<sup>3</sup>/TM3,Sb</i>	<i>P</i> -element insertion at <i>fru</i> locus. Homozygous defective courtship behaviour and male sterile.	Castrillon <i>et al.</i> , 1993; Goodwin <i>et al.</i> , 2000
<i>fru<sup>GAL4</sup>/TM3,Sb,Ser</i>	GAL4 knock-in at the <i>fru</i> locus by homologous recombination. Drives expression in all <i>fru</i> neurons. Homozygous courtship behaviour defective, partially fertile.	Stockinger <i>et al.</i> , 2005
<i>Df(3R)fru<sup>4-40</sup>/TM6B,Tb,Hu,e</i>	Chromosomal deletion of at least 70 kb covering <i>fru</i> locus, removing transcripts from P1 and P2 transcripts.	Anand <i>et al.</i> , 2001
<b>Cell Death Mutations</b>		
<i>Df(3L)XR38,ry/TM6B,Hu</i>	Small deletion covering the <i>reaper (rpr)</i> locus.	Peterson <i>et al.</i> , 2002
<i>Df(3L)H99/TM6B,Hu,ry</i>	Deletion covering cytogenetic locations 75C1-75C2; removes cell death genes <i>rpr</i> , <i>hid</i> and <i>grim</i> .	Abbott and Lengyel, 1991; White <i>et al.</i> , 1994; Grether <i>et al.</i> , 1995
<b>Homologous Recombination Stocks</b>		
<i>y*,w*,ey-FLP</i>	<i>P</i> -element insertion on X chromosome, used during the first stage of gene targeting by homologous recombination.	B.Dickson (unpub.)
<i>y*,w*;ey-FLP</i>	<i>P</i> -element insertion on 2 <sup>nd</sup> chromosome, used during the first stage of gene targeting by homologous recombination.	B.Dickson (unpub.)
<i>y*,w*,ey-FLP;Pin/CyO</i>	<i>P</i> -element insertion on X chromosome in a second chromosome balancer background, with <i>Pin</i> as a dominant marker for the other second chromosome; used during the first stage of gene targeting by homologous recombination.	B.Dickson (unpub.)

Strain/Genotype	Description	Reference
$y^*, w^*, ey\text{-FLP}; +; Ly/TM3, Sb$	<i>P</i> -element insertion on X chromosome, in a third chromosome balancer background, with <i>Ly</i> a dominant marker for the other third chromosome; used during the first stage of gene targeting by homologous recombination.	B.Dickson (unpub.)
$y^*, w^*/Y, hs\text{-}hid; Sco, hs\text{-}l\text{-}Scel, hs\text{-}FLP/CyO$	<i>P</i> -element insertions on the Y and second chromosomes; used during first stage of gene targeting by homologous recombination to excise the donor <i>P</i> -element from genome and create a double-strand break in the extrachromosomal DNA prior to integration at the desired locus.	B.Dickson (unpub.)
$w^*; +; hs\text{-}l\text{-}CreI, Sb$	<i>P</i> -element insertion on the third chromosome; used to create a double stranded break in the targeted locus to resolve the duplication created in the first stage of homologous recombination.	Rong <i>et al.</i> , 2002
<b>Sex Determination Transgenes</b>		
$w^{1118}; P\{w^{+mc}=UAS\text{-}tra.F\}2057; +$	Female-specific splice variant of the <i>tra</i> gene.	Ferveur <i>et al.</i> , 1995
<b>Balancer Chromosomes</b>		
$w^h; ap^{Xa}/CyO; +$	Strain isogenised to wild-type Canton-S strain. Second chromosome balancer ( <i>CyO</i> ) and dominant marker ( <i>ap</i> <sup>Xa</sup> ).	Flybase, 2007 S.F. Goodwin
$TM3, Sb, e/TM6B, Tb, Hu, e$	Strain isogenised to Canton S. Third chromosome balancers.	Flybase, 2007 S.F. Goodwin
<b>Isogenised Strains</b>		
<i>Canton-S (CS)</i>	Isogenic wild-type strain.	Flybase, 2007
$w^h$	<i>w</i> mutant isogenised to Canton-S.	Billeter, 2003
$w^{1118}$	<i>w</i> mutant used for microinjections	Flybase, 2007
$T\beta H^{M18}$	Null mutant in <i>tyramine-β-hydroxylase</i> gene ( <i>TβH</i> ), isogenized to Canton-S.	Monastirioti <i>et al.</i> , 1996

**Table 2.1-***Drosophila* stocks used in this study. 'w\*' or 'y\*' indicates that the precise allele of *w* or *y* is unknown.

### 2.1.2 Wild-Type Strain

Unless otherwise stated, the wild-type strain used in this thesis is Canton S (CS).

### 2.1.3 Sex Determination Mutant Genotypes

Null mutations in the *transformer* (*tra*) gene were obtained using heterozygous combinations of *tra*<sup>1</sup>/*Df*(3L)*st-J7* (as per Billeter *et al.*, 2006b). Null mutations in the *doublesex* (*dsx*) gene were obtained using heterozygous combinations of either *dsx*<sup>1</sup>/*Df*(3R)*dsx*<sup>15</sup> or *In*(3R)*dsx*<sup>23</sup>/*Df*(3R)*dsx*<sup>15</sup> (as per Villella and Hall, 1996). The *dsx*<sup>sw</sup> allele was always examined *in trans* to *dsx* deficiency stock *dsx*<sup>43</sup> (as per Waterbury *et al.*, 2000). Males and females were distinguished in *dsx* and *tra* null mutants using a *Bar-stone* marked Y-chromosome. *Fru*<sup>M</sup> mutants used for the examination of axonal morphology of the motor neurons innervating the direct flight muscles were *fru*<sup>3</sup> homozygous males. *fru* and *dsx* double mutants were of genotype *In*(3R)*dsx*<sup>23</sup>, *fru*<sup>3</sup>/*Df*(3R)*dsx*<sup>15</sup>, *fru*<sup>3</sup>. *fru*<sup>M</sup> and *fru*<sup>Δtra</sup> alleles were always examined *in trans* to *Df*(3R)*fru*<sup>4-40</sup> (as per Demir and Dickson, 2005).

### 2.1.4 Cell Death Mutant Genotypes

Null mutations in the *reaper* (*rpr*) gene were obtained using heterozygous combinations of *Df*(3L)*XR38*/*Df*(3L)*H99* (White *et al.*, 1994; Petersen *et al.*, 2002).

### 2.1.5 Rearing Conditions

*Drosophila* stocks were raised on ‘Glasgow’ medium. Stocks were maintained at room temperature in the laboratory or at 18°C. For genetic crosses and experiments, the flies were mated and their progeny raised either in an incubator or a designated fly room at 25°C with 12:12 hour light-dark cycles (8 am:8 pm).

Glasgow Medium: 10 g agar, 15 g sucrose, 30 g glucose, 35 g dried yeast, 15 g maize meal, 10 g wheat germ, 30 g treacle, and 10 g soy flour per litre of distilled water.

*Drosophila* adult females were allowed to lay eggs on apple juice agar plates for approximately 16 h, after which the embryos were collected from the plates, as described in 2.1.8.7.

**Apple Juice Agar:** 20 g agar, 26 g sucrose, 52 g glucose, 7 g dried yeast, and 9% (v/v) apple juice (The Organic Juice Company) per litre of distilled water.

For these media, distilled water was heated to boiling to dissolve the agar. The remaining reagents were then added and allowed to dissolve. The media was allowed to cool to 60°C before being supplemented with Nipagin M (4-hydroxybenzoic acid methylester, 10% (w/v) in absolute ethanol) to inhibit fungal growth.

## **2.1.6 *Drosophila* Microinjection**

### **2.1.6.1 DNA Solution**

Plasmids for microinjection were purified from *E. coli* DH5 $\alpha$  (Table 2.4), using the Plasmid Maxi Kit (QIAGEN), following the standard protocol. The helper plasmid pUCHS $\pi$  $\Delta$ 2-3 (Laski *et al.*, 1986) was co-injected with the non-autonomous *P*-element transformation vector containing the transgene to provide a source of transposase (ratio of helper:non-autonomous *P*-element = 1:5). The mixture was then injected at an approximate concentration of 100  $\mu$ M/ml.

### **2.1.6.2 Microinjection**

*Drosophila* embryos of genotype  $w^{1118}$  were microinjected with the DNA solution described above using standard microinjection procedures by EMBL *Drosophila* microinjection service, or Genetic Services Inc. (Cambridge, MA).

### **2.1.6.3 Selecting *P*-element Transformants**

Larvae were returned by post approximately 48 hr after injection. They were allowed to develop into adults in a food vial at 25°C. Single adults were collected after eclosion and mated with  $w^{1118}$  flies of the opposite sex. The progeny of these crosses were screened for the presence of orange eyes, indicating *P*-element integration. The *P*-elements used in this study all bear the *mini-white* (*mw*) gene, which confers varying degrees of eye colour to flies when in a  $w^{1118}$  genetic background.

#### 2.1.6.4 Determination of Chromosomal Insertion

*P*-element lines were crossed to different balancer chromosomes with dominant genetic markers to determine the chromosome of insertion. Since all the *P*-elements used in this work contain the *mw* gene as a marker, all genetic crosses were carried out using flies with balancer chromosomes in a *w* background. The *P*-elements were followed using the eye colour conferred by the presence of the *mw* transgene.

To determine whether an insertion occurred on the second chromosome, flies heterozygous for the presence of the *P*-element were crossed to  $w^h;ap^{xa}/CyO$ . The  $w^h;+/CyO$  progeny with orange eyes were then backcrossed to  $w^h;ap^{xa}/CyO$ . Insertion on the second chromosome was determined by the absence of  $w^h;ap^{xa}/CyO$  progeny with orange eyes.

Similarly, insertions on the third chromosome were determined by crossing the *P*-element line to  $w^{1118};+;TM3,Sb,e/TM6B,Tb,Hu,e$  and by following the same logic as for the second chromosome. For insertion on the first chromosome (X), males carrying the *P*-element insertion were crossed to  $w^h$  females. Integration on the first chromosome was determined by the absence of male progeny with orange eyes (inherited the *mw* marker). After excluding insertions on the first, second and third chromosome, the insertion was determined to have occurred on the fourth chromosome. No insertions were found on the Y chromosome.

Once the chromosome bearing the *P*-element was determined, the insert was balanced with the appropriate chromosomal balancer. Homozygous viable lines were kept both as homozygous stocks and as heterozygotes with an appropriate balancer chromosome. Lethal insertion lines were kept as heterozygotes with the appropriate balancer chromosome.

#### 2.1.7 Outcrossing the *P* Insertion Lines into a Canton-S

##### **Background**

All the *P*-element lines to be used in behavioural assays were outcrossed to isogenize their genetic background with that of a *Canton-S* wild type strain. White-eyed virgin females of the *Canton-S* ( $w^h$ ) strain were collected and crossed to orange-eyed males of the *P*-element insertion line of interest. The



*mini-white* marker was used to follow the transgene throughout the outcrossing procedure. At the F1 stage, one orange-eyed virgin female was crossed to three *Canton-S* ( $w^h$ ) males. At the F2 stage, one orange-eyed virgin female was crossed again to *Canton-S* ( $w^h$ ) males. This process was repeated for at least five generations. After outcrossing, a single virgin female was collected and crossed to three sibling males, and the homozygous progeny from this cross were used to generate the final outcrossed stock.

## **2.1.8 Dissection Techniques**

### **2.1.8.1 Whole Mount Dissection of the Adult CNS**

Flies were aged for 5-7 days before dissecting, unless stated otherwise. After light CO<sub>2</sub>-anaesthesia, flies were immersed in ethanol for 5 s. They were then transferred into phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na<sub>2</sub>HPO<sub>4</sub>, 2 mM KH<sub>2</sub>PO<sub>4</sub>) in a dissecting dish lined with silicon gel (Sylgard, Dow-Corning). Their wings and legs were removed using dissection forceps. The brain was isolated by first removing the proboscis, and then by tearing the head capsule in half by pulling at the lower corner of each eye. The brain was left attached to the rest of the body. The abdomen was then severed using dissection scissors. The forceps were used to cut the thoracic cuticle transversely from anterior to posterior above the base of the legs. The upper part of the thorax containing the flight muscles was then removed, and the legs were cut off using dissection scissors. The oesophagus was removed to reveal the dorsal part of the ventral nerve cord (VNC), and the VNC gently lifted from the thorax. All tracheae attached to the isolated CNS were gently removed with the forceps.

### **2.1.8.2 Whole Mount Pupal CNS**

Wandering 3<sup>rd</sup> instar larvae make their way to the side of the vial, where they eventually evert their spiracles and stop moving (Bainbridge and Bownes, 1981). These pre-pupae were collected and sexed by the size of their gonads (Ashburner, 1989).

Individuals were then transferred to a 50 mm Petri dish humidified with a wet tissue, and the pupae were aged at 25°C for 48 hours.

Pupae at the desired stage were transferred to PBS for dissection. The operculum was first removed using forceps. The rest of the pupal case was then gently peeled off to reveal the pupa. A slit was made with the forceps along the dorsal part of the thorax, extending to just below the head. A Gilson P20 micropipette fitted with a yellow tip was used to jet 20 µl of PBS in and out of this opening, which cleared the inside of the thorax and allowed the visualization of the pupal CNS. The abdomen was then severed using forceps. Eventually, the PBS current dislodged the CNS from the head capsule, and the whole CNS was isolated.

### **2.1.8.3 Whole Mount Larval CNS**

Wandering 3<sup>rd</sup> instar larvae were collected with a paintbrush and transferred to PBS. Larvae were sexed according to the size of their gonads (Ashburner, 1989). Forceps were then used to grab the mid-part of the body. The forceps were pulled apart, and the brain lifted out of the remains.

### **2.1.8.4 Imaginal Discs**

Wandering 3<sup>rd</sup> instar larvae were collected with a paintbrush and transferred to PBS. Larvae were sexed as described (Ashburner, 1989). Forceps were used to pull apart the larva, as described in 2.1.8.3. Following the removal of the brain, the anterior half of the larva was removed, with imaginal discs still attached.

### **2.1.8.5 Direct Flight Muscle Dissection**

Flies were aged for 5-7 days prior to dissection. After light CO<sub>2</sub>-anaesthesia, flies were immersed in ethanol for 5 s and then transferred to PBS. Their wings, legs, head and abdomen were removed using dissection scissors. One blade of the dissection scissors was inserted into the anterior opening of the thorax, and a dorsal cut was made from anterior to posterior. The same cut was repeated on the ventral side of the thorax. The indirect flight muscles (dorsal longitudinal and dorsoventral muscles) and associated trachea were removed using dissection forceps, exposing the direct flight muscles (DFMs). Excess cuticle was removed

using dissection scissors until only a small portion of the thorax directly surrounding the DFMs near the wing hinge remained.

#### **2.1.8.6 Fluorescent Labelling of Direct Flight Muscles**

Direct flight muscle preparations were fixed for 30 min in 4% (w/v) paraformaldehyde (in PBS). Preparations were then washed three times for 10 min per wash in PBS, followed by a single wash in PBT (PBS, 0.5% (v/v) Triton-X). The preparations were then incubated with 1  $\mu$ M TRITC (tetramethylrhodamine B isothiocyanate)-conjugated phalloidin (Sigma) for 20 min. The preparations were given three 10 min washes with PBT, followed by three 10 min washes in PBS before being mounted in VectaShield (Vector Lab) on a slide.

#### **2.1.8.7 Embryo Collection**

Approximately 50 females and 25 males were pooled in an inverted plastic beaker and allowed to lay eggs on a plastic Petri dish containing apple juice agar medium with a dab of yeast paste (dried yeast in distilled water, heat inactivated) covering the open bottom of the beaker. Flies were allowed to lay overnight at 25°C on the plate, and the plate was removed the following morning for collection. Embryos were detached from the egg laying plate using a paintbrush and a stream of distilled water, and collected in a fine-mesh sieve.

### **2.2 Visualization Techniques**

#### **2.2.1 X-Gal Staining**

Dissected CNSs, appendages, and reproductive organs were fixed for 5 min in 4% (w/v) paraformaldehyde in PBS, and washed three times in PBS for 15 min each, then incubated in X-Gal staining solution at 37°C for varying amounts of time. After staining, the specimens were washed twice in PBS and cleared through a glycerol series of 30%, 70% and 90% (v/v in PBS). The specimens were mounted in 90% glycerol on a microscope slide. Two coverslips (22 mm square, No. 1 thickness, BDH) were placed on each side of the mounted preparations to act as spacers before placing a coverslip on top of the preparation.

X-Gal Staining solution: 0.2 M NaPi (pH 7.4), 5 M NaCl, 1 M  $\text{MgCl}_2$ , 50 mM K $\cdot$ Ferro-CN, 50 mM K $\cdot$ Ferri-CN, 10% (v/v) Triton X-100 per 10 ml of distilled water. 0.9 ml aliquots of this solution were made and stored at  $-20^\circ\text{C}$ . A given aliquot was heated to  $60^\circ\text{C}$ , centrifuged to remove particulate matter, and immediately prior to use, 0.1 ml of 20 mg/ml 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside (X-Gal) in dimethylformamide (DMF) was added. Specimens were examined under an Axiophot 2 microscope (Zeiss) using either bright field microscopy or Differential Interference Contrast (DIC). Images were captured using an Axiocam (Zeiss) CCD camera connected to a PC.

### **2.2.2 Immunofluorescence**

Samples to be stained with anti-Fru<sup>M</sup>, anti-Dsx or anti- $\beta$ -Gal were dissected in PBS and fixed in 2% (w/v) paraformaldehyde (in PBS) for 30 min on ice. They were washed three times in PBS for 15 min each, three times in PBT (PBS, 0.3% (v/v) Triton X-100), and incubated for 1 hour in PTN (PBT, 5% (v/v) normal goat or sheep serum (Scottish Diagnostics and Molecular Probes)). The specimens were then incubated at  $4^\circ\text{C}$  overnight in PTN containing the primary antibody at the appropriate dilution (see Table 2.2). The samples were then washed four times in PBT for 30 min per wash, and incubated in PTN containing the appropriate species-specific Alexa fluor-conjugated secondary antibody (see Table 2.3) for 3 hours at room temperature. Samples were then washed four times in PBT for 30 min per wash and three times in PBS for 10 min per wash. Finally, specimens were mounted in VectaShield (Vector Lab) on polylysine-treated microscope slides (BDH).

Samples to be stained with all other antibodies except anti-octopamine and anti-glutamate were fixed in 4% (w/v) paraformaldehyde (in PBS) for 30 min at room temperature. The remaining steps were as above.

Samples to be stained with anti-octopamine were fixed for 30 min on ice in 4% (w/v) EDAC (1-(3-dimethylaminopropyl-3-ethylcarbodiimide,hydrochloride)) in PBS, with all remaining steps as described above.

Samples to be stained with anti-glutamate were fixed for 30 min on ice in 4% (w/v) paraformaldehyde with 0.2% (w/v) glutaraldehyde (in PBS). All remaining steps were as described above.

Embryos were collected as described in section 2.1.8.7, and dechorionated by placing the sieve into a 50% solution of bleach in distilled water for exactly 3 min, after which the embryos were washed thoroughly with distilled water to remove all traces of bleach. The embryos were then transferred to 5 ml heptane in a glass container using a paintbrush, and 5 ml of 4% (w/v) paraformaldehyde (in PBS) was added, and the solution was shaken vigorously. The embryos were fixed for 10 min at room temperature. After fixation, the aqueous bottom layer was completely removed, and a 5 ml solution of 95% methanol/5% EGTA (ethylene glycol tetraacetic acid, pH 8.0) was added to the glass container, and swirled gently. De-vitellinized embryos sank to the bottom of the container. Embryos were then transferred to a clean 1.5 ml Eppendorf tube containing 1.0 ml of PBS. After the embryos had fallen to the bottom of the tube, most of the PBS was removed and several drops of PAT (PBS, 1% Triton-X, 0.1% bovine serum albumin (BSA)) were added. The embryos were then washed an additional three times with PAT, followed by two washes in PBS. To mount, the PBS was removed and VectaShield (Vector Lab) was added, and the embryos were transferred to a polylysine slide for viewing.

Antibody	Description	Dilution	Source/Reference
Anti-Fru <sup>M</sup>	Rat, polyclonal against male-specific 101 aa domain of Fru <sup>M</sup> .	1:300	Lee <i>et al.</i> , 2001
Anti-Fru <sup>M</sup>	Rabbit, polyclonal against male-specific 101 aa domain of Fru <sup>M</sup> .	1:400	Billeter <i>et al.</i> , 2006b
Anti-Dsx	Rat, polyclonal against common DM DNA-binding domain of Dsx.	1:300	Cho and Wensink, 1996; Lee <i>et al.</i> , 2002
Anti-βGal	Rabbit, polyclonal against <i>E.coli</i> β-Galactosidase.	1:1000	Cappel, ICN
Anti-Horseradish Peroxidase	Cy3 conjugated affinity purified goat polyclonal against HRP.	1:300	Jackson ImmunoResearch Laboratories Inc.
Anti-5HT	Rabbit, polyclonal against 5-Hydroxytryptamine.	1:500	Sigma

Antibody	Description	Dilution	Source/Reference
Anti-Glutamate	Rabbit, polyclonal against L-glutamate-glutaraldehyde-BSA.	1:100	Chemicon International
Anti-Octopamine	Rabbit, polyclonal against octopamine conjugated to KLH.	1:100	Chemicon International
Anti-Repo	Mouse, monoclonal against glial cell marker Repo.	1:50	Developmental Studies Hybridoma Bank, University of Iowa
Anti-mCD8	Rat, monoclonal against mouse lymphocyte marker CD8 $\alpha$ -subunit.	1:20	Caltag Laboratories
Anti-Green Fluorescent Protein	Rabbit, polyclonal against GFP.	1:1000	Molecular Probes Inc.

**Table 2.2**-Primary antibodies used in this study.

Antibody	Description	Dilution	Source/Reference
Anti-rat IgG-Alexa Fluor 488	Goat, polyclonal against rat IgG, conjugated to Alexa Fluor 488.	1:600	Molecular Probes, Inc.
Anti-rabbit IgG-Alexa Fluor 488	Goat, polyclonal against rabbit IgG, conjugated to Alexa Fluor 488.	1:600	Molecular Probes, Inc.
Anti-rabbit IgG-Alexa Fluor 546	Goat, polyclonal against rabbit IgG, conjugated to Alexa Fluor 546.	1:600	Molecular Probes, Inc.

**Table 2.3**-Secondary antibodies used in this study. IgG stands for Immunoglobulin Gamma.

### 2.2.3 Confocal Microscopy

Stained whole mounts were examined with a Zeiss LSM 510 Meta confocal microscope equipped with x10, x20, x40 (oil immersion), and x63 (oil immersion) objectives. The Ar/HeNe lasers of the Zeiss can excite fluorochromes with the following wavelengths in the visible spectrum: 458 nm, 476 nm, 488 nm, 514 nm,

543 nm and 633 nm. Alexa fluor-488-conjugated secondary antibodies were excited with the 488 nm line, and emit in the green spectrum. TRITC-conjugated phalloidin and the Alexa Fluor 546-conjugated secondary antibodies were excited with 543 nm line, and emit in the red spectrum.

For double-labelling, the 488 nm and 543 nm lines were used sequentially for each optical section through the sample to excite both fluors individually and avoid bleed-through. Stacks of optical sections were generated at 1.7  $\mu\text{m}$  spacing.

### **2.2.4 Image Processing**

Digital images generated by the confocal microscope or by the digital cameras were saved and stored as 'tiff' (Tagged Image File Format) files.

#### **2.2.4.1 Processing of Images for Figures**

Pseudocolours were added to images generated by confocal microscopy using the software 'Image J' (National Institutes of Health, Washington) with the 'Look Up Table' function ('LUT'). A green LUT was applied to images obtained from samples stained with Alexa fluor 488-conjugated secondary antibodies; and a red LUT was applied to samples stained with either Alexa fluor 546- or TRITC-conjugated secondary antibodies. LUTs replaced the greyscale value produced by the confocal microscope with colours. Three-dimensional (3D) reconstruction of confocal stacks were performed using the Image J software using the 'Maximum Projection' function, which is a flattened composite of the brightest pixels found through the vertical (or *Z*) axis of a given number of optical sections. Pseudocoloured 3D reconstructions or individual optical sections for co-localization experiments were merged using the Image J 'RGB Stack Merge' plugin and saved as 'tiff' files.

Images obtained by conventional microscopy were processed using Adobe Photoshop 7.0. White balance was adjusted using the 'Level' Function in the 'Image' menu. Brightness and contrast were also adjusted if necessary. Processed images were saved as 'tiff' files.

Figures were assembled using Adobe Illustrator 10 (Adobe Systems Inc., San Jose, CA).

#### 2.2.4.2 Cell Counts

For cell counts, stacks of optical sections obtained by confocal microscopy were opened using the LSM Image Browser 5 program. Each optical slice was examined in sequence, and all labelled nuclei per slice were marked manually using the ‘Overlay’ function. Any marked nucleus appearing in more than one consecutive slice was counted only once. After all optical slices were examined, the number of marked nuclei were counted. All cell counts were performed on slides coded by an unbiased third party.

### 2.3 Bacterial Protocols

#### 2.3.1 Strains and Plasmids

The only *Escherichia coli* strain used in this study was DH5 $\alpha$  (Hanahan, 1983); its genotype is given below.

Strain	Genotype
DH5 $\alpha$	F <sup>-</sup> , $\phi$ 80d <i>lacZ</i> $\Delta$ M15 $\Delta$ ( <i>lacZ</i> YA- <i>argF</i> )U169 <i>deoR</i> <i>recA1</i> <i>endA1</i> <i>hsdR17</i> (r <sub>k</sub> <sup>-</sup> , m <sub>k</sub> <sup>+</sup> ) <i>phoA</i> <i>supE44</i> $\lambda$ <i>thi-1</i> <i>gyrA96</i> <i>relA1</i>

**Table 2.4-***E.coli* strain used in this study.

Plasmids used in this study, other than those whose construction is described elsewhere in this thesis, are listed below.

Plasmid	Size (bp)	Description	Antibiotic Resistance	Source or Reference
PCR <sup>®</sup> 2.1-TOPO <sup>®</sup>	3900	Sub-cloning vector for cloning PCR products using topoisomerase.	Ampicillin	Invitrogen
pZER0-2	3297	Sub-cloning vector. Allows direct selection of recombinants via disruption of a lethal gene inserted in the multiple cloning site of the plasmid.	Kanamycin	Invitrogen



Plasmid	Size (bp)	Description	Antibiotic Resistance	Source or Reference
pMartini	~6000	Derivative of pBluescript with an expanded polylinker, and containing GAL4 coding sequence.	Ampicillin	Gift from N. Brown (Cambridge University)
pStingerII	12443	pCaSper- <i>lacZ</i> derivative. The <i>lacZ</i> gene was replaced with eGFP flanked by su(Hw) binding sites to insulate it.	Ampicillin	Barolo <i>et al.</i> , 2000
pED22	~12	Vector for gene targeting by homologous recombination. Contains multiple cloning sites, <i>P</i> -element transposition sequences, a mini-white marker gene and the I- <i>SceI</i> and I- <i>CreI</i> recognition sites.	Ampicillin	Gift from B. Dickson, unpub.
BACR11D12	189596	pBACe3.6 vector carrying a <i>Drosophila melanogaster</i> chromosome fragment spanning cytological positions 84D-84E.	Chloramphenicol	Celniker <i>et al.</i> , 2001
BACR07M14	181950	pBACe3.6 vector carrying a <i>Drosophila melanogaster</i> chromosome fragment spanning cytological positions 84F-84F.	Chloramphenicol	Celniker <i>et al.</i> , 2001
pUCHS $\pi\Delta$ 2-3	~7477	Helper plasmid for germ-line transformation.	Ampicillin	Laski <i>et al.</i> , 1986

**Table 2.5**-Plasmids used in this study.

### 2.3.2 Culture Media

SOC: 2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl<sub>2</sub>, 10 mM MgSO<sub>4</sub>, 20 mM glucose, pH 7.0

L-Broth: 10 g Bacto-tryptone (Difco), 5 g yeast extract (Difco), and 10 g NaCl per litre of water; pH was adjusted to 7.0 with NaOH.

L-Agar: As above with the addition of Bacto-agar (Difco) to 1.5% (w/v).

All culture media were sterilised by autoclaving at 120°C for 15 min.

### **2.3.3 Antibiotics and Indicators**

When necessary, antibiotics were added to culture media for plasmid selection. Antibiotics were added to broth at room temperature and to molten agar at 50°C. Final concentrations of antibiotic used were as follows: 100 µg/ml for ampicillin, 25 µg/ml for kanamycin and 20 µg/ml for chloramphenicol. All antibiotics were purchased from Sigma.

X-Gal and Isopropyl-β-D-thiogalactopyranoside (IPTG) were added to L-Agar to detect recombinant clones by α-complementation (blue/white selection). 40 µl of X-Gal (20 mg/ml in DMF) and 4 µl of IPTG (200 mg/ml in sterile distilled water) were spread on the surface of a solidified L-Agar Petri dish (98 mm diameter) (Sambrook and Russell, 2001).

### **2.3.4 E. coli Transformation Techniques**

#### **2.3.4.1 Preparing Competent E. coli Using Chemical Treatment**

The Hanahan method (Hanahan *et al.*, 1995) was used for the generation and long-term storage of high efficiency competent bacteria. This method yields high efficiency of transformation in the range of 10<sup>8</sup> transformants/µg of plasmid pUC19.

#### **2.3.4.2 Transformation**

50-150 ng of DNA in a maximum volume of 1 µl was added to 50 µl of competent cells suspension and incubated on ice for 15 min. The cells were heat shocked at

42°C for 90 sec, cooled on ice for 2 min and supplemented with 900 µl of SOC. The suspension was incubated at 37°C with vigorous shaking for 1 hr, plated onto L-Agar Petri dishes with the appropriate antibiotics and indicator, and incubated overnight at 37°C to select for transformants.

#### **2.3.4.3 Preparation of Electrocompetent Cells**

DH5α were grown in 400 ml of L-Broth at 37°C with shaking until they reached an O.D.<sub>600</sub> between 0.4 and 0.5. The cells were chilled on ice for 30 min and centrifuged at 4000 rpm for 10 min at 4°C. The cells were resuspended in 200 ml of ice-cold 10% (v/v) glycerol (in sterile distilled water), centrifuged, and resuspended in 100 ml of 10% (v/v) glycerol a further two times. Finally, the cells were centrifuged and resuspended in a final volume of 1 ml of 10% glycerol. Cells were divided into 100 µl aliquots and stored at -70°C.

#### **2.3.4.4 Precipitation and Purification of DNA for Electroschock Transformation**

DNA solutions were desalted prior to electroporation, according to the method of Zhu and Dean (1999). 5 µl of ligation product was mixed with 1 µl of 1 µg/µl yeast tRNA (Gibco BRL) and 14 µl sterile distilled water. 50 µl of 100% cold ethanol was added to precipitate the DNA. After 30 min at -20°C, the sample was centrifuged at 14000 g for 10 min at 4°C. The pellet was washed once with 100 µl of 70% ethanol (in sterile distilled water), centrifuged for 5 min at 14000 g, allowed to air dry, and resuspended in 2 µl of 5 mM Tris-HCl (pH 8.5).

#### **2.3.4.5 Electroschock Transformation of *E. coli***

1 µl of desalted DNA solution was added to 50 µl of electrocompetent cells. Individual aliquots of cells and DNA were transferred into pre-chilled electroporation cuvettes (0.2 cm gap electrode, BioRad). The cells were then electroschocked at 2.5 kV in a MicroPulser™ (BioRad). 950 µl of SOC medium was added into each cuvette immediately following electroschock. The cell suspension was quickly transferred to a fresh microcentrifuge tube and incubated for 1 hour at 37°C with vigorous shaking. 100 µl of the cell suspension was then plated onto L-agar containing the appropriate antibiotic and indicators, and incubated overnight at 37°C to select for transformants.

### **2.3.5 Isolation of Bacterial Artificial Chromosome (BAC) and Plasmid DNA from *E. coli***

#### **2.3.5.1 Small-Scale Isolation of BAC and Plasmid DNA**

DNA was purified from 5-10 ml of overnight culture grown in L-Broth at 37°C with shaking in the presence of the appropriate concentration and type of antibiotic. BAC and plasmid DNA were both isolated using the alkaline lysis method (as described in Sambrook and Russell, 2001). Plasmid DNA for sequencing and cloning was isolated using the QIAprep spin miniprep kit (QIAGEN) according to the manufacturer's instructions.

#### **2.3.5.2 Large-Scale Isolation of Ultra-Pure BAC and Plasmid DNA**

Large-scale preparations of BAC and plasmid DNA were performed using the Maxiprep kit (QIAGEN) according to the manufacturer's instructions.

## **2.4 General Molecular Biology Protocols**

### **2.4.1 Restriction Digestion of DNA**

DNA was digested in the appropriate buffer for the enzyme(s) according to manufacturer recommendations (New England Biolabs). 2 units of enzyme were used per µg of DNA to ensure complete restriction of DNA. Digests were carried out at 37°C for 1-2 hours for plasmid DNA, and 4 hours for genomic DNA (where half of the enzyme was added at the beginning of the digest and the other half after 2 hours).

### **2.4.2 DNA Gel Electrophoresis**

DNA fragments were resolved on agarose (Boehringer Mannheim) gels. The DNA solution was mixed with loading buffer and loaded into agarose gels containing 1 X TAE (40 mM Tris/AcOH (pH 8.2), 20 mM NaOAc, 1 mM EDTA). A range of agarose concentrations (0.6%-2.0% (w/v)) were used depending on the size of the fragments to be resolved (Sambrook and Russell, 2001). The gels were run

from 1 hour to overnight at voltages ranging from 25 mV to 120 mV. DNA size markers were the 100 bp or the 1 kb ladders (New England Biolabs).

### **2.4.3 Visualization and Photography of Gels**

DNA was visualized by UV-induced fluorescence on either a short wave (254 nm) or long wave (365 nm) transilluminator after staining the agarose gels in an ethidium bromide (EtBr) solution (0.5 µg/ml) in a shaker tray for 40 min. For very large DNA fragments, the DNA was visualized on a light box after staining the agarose gels for 1 hour with crystal violet (0.0025% (w/v) in distilled water (Yang *et al.*, 2001), and de-staining for 20 min in distilled water. Gels were photographed using a Polaroid camera loaded with Polaroid 667 film and fitted with a Kodak Wratten filter No. 23A.

### **2.4.4 Gel Extraction of DNA Fragments**

Size-selected DNA fragments were excised from agarose gels using a clean razor blade, on the long wave (365 nm) UV transilluminator. Purification of the DNA fragment from agarose, salt and enzymes was carried out with the QIAquick gel extraction kit (QIAGEN), or the QIAEXII Gel Extraction Kit (for fragments >10 kb)(QIAGEN) following manufacturer's instructions.

### **2.4.5 Subcloning DNA Fragments into Plasmid Vectors**

The insert to be sub-cloned and the target plasmid vector (Table 2.5) were digested with restriction enzymes as described in 2.4.1. When the plasmid vectors were cut with a single enzyme, the overhanging 5' or 3' nucleotides triphosphate were dephosphorylated to prevent vector re-ligation (except in the case of pZErO-2, which did not require such treatment). 1 µl of Antarctic Phosphatase (New England Biolabs) was added directly to the completed plasmid digest reaction and incubated at 37°C for 1 hour. The alkaline phosphatase was then heat-inactivated for 15 min at 68°C. Both insert and vector were size fractionated and gel purified as described in 2.4.2 and 2.4.4.

### **2.4.6 Ligation**

Purified inserts and vectors were mixed at a ratio of 3:1 (insert:vector) for cohesive end ligation and 1:1 for blunt end ligation. The DNA mix was combined with 1 µl (1 Weiss Unit) of T4 DNA ligase and 4 µl of 5x T4 DNA ligase buffer (New England Biolabs) in a total volume of 20 µl. The ligation mixture was incubated overnight at 4°C and transformed as described in 2.3.4.2 and 2.3.4.5. Transformants were selected on L-Agar plates in the presence of the appropriate antibiotics and indicators.

### **2.4.7 Identification of Plasmids Containing Large Inserts**

In order to identify plasmids containing large inserts, the ‘cracking’ technique was employed. Large numbers of transformant colonies were chosen, and 700 µl mini-cultures were grown from each transformant in 24-well plates overnight at 37°C. Each mini-culture was spun for 1 min at 12000 g, and the supernatant was removed. 40 µl of cracking buffer (50 mM Tris pH 6.8, 2 mM EDTA, 0.4 M sucrose, 1% (w/v) SDS, 0.01% (w/v) bromophenol blue, 0.1 mg/mL RNase A) was added to the pellet, and the pellet was resuspended by pipetting up and down quickly several times. The resuspended cells were incubated in the cracking buffer for 15 min, and then spun at 12000 g for 5 min. The supernatant was then loaded onto a 1% agarose gel and run at 100 mV for 1 hour. The gel was stained with ethidium bromide and photographed, as described in 2.4.3. Plasmids containing large inserts travelled a shorter distance in the agarose gel than plasmids containing smaller or no inserts. Mini-cultures corresponding to the plasmids potentially containing large inserts were used to start 5-10 ml overnight cultures, and these plasmids were then purified (as described in section 2.3.5.1) for diagnostic restriction digests (as described in 2.4.1).

### **2.4.8 Purification of Genomic DNA**

Fly genomic DNA was prepared by a modification to the procedure described in Hamilton and Zinn (1994). 0.04 g of flies (approx. 30 flies) were frozen on dry ice and homogenized with a micro-pestle in 200 µl of grinding buffer (5% sucrose, 80 mM NaCl, 100 mM Tris, pH 8.5, 0.5% SDS, 50 mM EDTA). The homogenate was then incubated at 70°C for 30 min, supplemented with 35 µl of

8 M KOAc, transferred to ice for 30 min and centrifuged at 12000 g for 10 min at 4°C. The supernatant was then transferred into a clean tube. Excess protein was extracted from the solution using a phenol:chloroform:isoamyl alcohol extraction (25:24:1; Sigma) (as described in Sambrook and Russell, 2001). The DNA was then precipitated by adding 150 µl of isopropanol to the supernatant and allowed to stand at room temperature for 5 min. The genomic DNA was pelleted by centrifugation at 12000 g for 15 min at 4°C. The pellet was rinsed with 500 µl of 70% EtOH and allowed to dry at room temperature. The pellet of genomic DNA was resuspended in 200 µl of TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) with 0.1 mg/ml of RNase A (Sigma).

#### ***2.4.9 Rapid Single Fly DNA Isolation for PCR***

Approximately 15 flies were homogenized in a 1.5 ml microcentrifuge tube with a micropestle in 50 µl of homogenization buffer (10 mM Tris-HCl, pH 8.3, 1 mM EDTA, 25 mM NaCl, 200 µg/ml Proteinase K (QIAGEN), in sterile distilled water). Genomic DNA was isolated from this homogenate using the QIAGEN Dneasy Blood and Tissue Kit, according to manufacturer's instructions (QIAGEN). 2 µL of the DNA was then used for PCR as described in 2.4.11.

#### ***2.4.10 Quantification of Nucleic Acids***

The amount of DNA in a sample was estimated by taking Optical Density (O.D.) measurement at a wavelength of 260 nm using a spectrophotometer. An  $O.D._{260}=1$  corresponded to ~50 µg/ml of double stranded DNA.

Estimation of DNA concentration in an agarose gel was also done by comparing a given band with the 3 kb band of the 1 kb ladder (New England Biolabs), which contains approximately 125 ng of DNA when ladder is prepared according to manufacturer's instructions.

#### ***2.4.11 Polymerase Chain Reaction (PCR)***

*Taq* polymerase (New England Biolabs) was used for diagnostic PCR, and Phusion high fidelity DNA polymerase (New England Biolabs) was used for applications requiring high fidelity amplification. The PCR mix was set according to manufacturer's instruction. PCR was carried out in a 'Robocycler Gradient 40'

thermal cycler (Stratagene). For PCR using *Taq* polymerase (New England Biolabs) the conditions were the following: initial denaturation- 5 min at 95°C; denaturation- 50 sec at 94°C, annealing- 40 sec 50-60°C, extension- 1 min at 72°C, where denaturation through extension was repeated for 29 cycles, followed by a final extension at 72°C for 5 min. For high fidelity PCR with Phusion DNA Polymerase (New England Biolabs) the conditions were the following: initial denaturation step- 30 sec at 98°C; denaturation- 15 sec at 98°C, annealing- 30 sec at 50-60°C, extension- 15 sec per kb at 72°C, where denaturation through extension was repeated for 29 cycles, followed by a final extension at 72°C for 10 min.

Primer Name	Sequence (5'-3')	T <sub>m</sub> (°C)
DsxGAL4HRBamHI1F	GGATCCCACTAAACGAGAATCAAAAAC	52.7
DsxGAL4HRXbaI1R	TCTAGAGAGAAAGACCCTGCTGGCTATCAG	64.8
DsxGAL4HRAvrII2Fa	CCTAGGCTTAAAGTGGCTCTTCGGAGG	60.4
DsxGAL4HRAvrII2Fb	GTGGCTCTTCGGAGGAATTATTC	63.4
DsxGAL4HRAvrII2Fc	TTAAAGTGGCTCTTCGGAGG	59.4
DsxGAL4HRBamHI2R	GGATCCGATTCCAGCTTCTGATATCCTA	55.3
DsxGAL4HRNotI4F	GCGGCCGCACAAAACCTTTCACCCAACTCAATA	59.0
DsxGAL4HRNotI4R	GCGGCCGCTTGTCTGCCAGGGGTTTTTCAG	65.6
HRISeq1	CGTCTAAAGGTGGCATAAATACGC	63.0
HRISeq2	TGCTTTCTCCCAGTTGATG	57.3
HRISeq3	CAAGAGCAAAGCACACCTC	57.1
HRISeq4	AAAGTTGAAGCGAAGGCG	59.1
HRISeq5	CACATTTTTTATGCTCGCCC	60.5
HRISeq6	GCAACAGAGAGTTACGGAGAG	56.8
HRISeq7	TACCGAATCCGCTCCAGTTC	62.4
HRISeq8	TACCGCCAGTTGTAGATGG	57.1
HRISeq9	TGCCATTTGCGTCATCCGTC	67.6
HRISeq10	ATAAGCAGGGAAACAGTGG	55.2
HRISeq11	AAACGAGTGCAGCATCCAACC	67.8
HRISeq1	GATACTGGCTTTCGTCTATGTC	55.7
HRISeq2	ACCCATTTCCAATAGCCC	57.2
HRISeq3	GTTTCACAGTTACAGTCTTGGG	56.4
HRISeq4	ACACACACCATCGCACATCC	62.9
HRISeq5	ATGTAAAGGGGCGTGGTTG	60.8
HRISeq6	GCTGAATAAGAGTGGAGTGC	55.1
HRISeq7	CTTGTCCACTTTCTCCACAC	55.6
HRISeq8	GCAACTACCTAACAAAGGAGC	55.9
HRISeq9	TGGCTGGCGTTGTTGTTGTC	65.5
HRISeq10	TAACAAGGGACCAAGGAAG	55.2
HRISeq11	TTTTGCTTCTGCTGCTGCTGACCG	71.6
HRISeq12	TGATTCAGCACCGATAGC	55.7
HRISeq13	CCCTTTACATTGCTTGGACTC	58.7
HRISeq14	GCGTCTAAGTTTATGTAACCGTGC	61.2
HRIVSeq1	AAGATTCGTCTCTGGGAGC	56.9



Primer Name	Sequence (5'-3')	T <sub>m</sub> (°C)
HRIVSeq2	CGACCAGATGAAAATCCC	56.3
HRIVSeq3	TATCCGATTCCCTTGGGAC	55.8
HRIVSeq4	GCTAACTAACCCGTCTTCAAAC	57.2
HRIVSeq5	TGTGTAACAAACGGACTGG	55.4
HRIVSeq6	TCACAAAGTCAAGGTAACCG	56.3
HRIVSeq7	GGTGGCAATCATTTAGTGG	56.4
HRIVSeq8	TGTTGACAGCCAGTGTATCC	57.1
HRIVSeq9	TCGTAGTCGTAGTTTGTGCTG	56.7
HRIVSeq10	AAGTGCCAAGTGCCTAACCTCG	64.8
HRIVSeq11	TGGCGGCACTAAATGTTC	58.2
dsx-GAL4JSeq	GCTGAATAAGAGTGGAGTGC	55.1
dsx-pStingJSeq	TCGCTCTCTCGCTCTCTAAC	57.7
dsx-dsxJFSeq	AGTTGAAGCGAAGGCGTTTC	61.8
dsx-dsxJRSeq	TGCTGAGGAGTCAAACGG	58.4
G0-back	CTTCCGATGATGATGTTCGCAC	55.0
G0-for	CAAGTGCTCCAAAGAAAAACCG	66.5
pMGS-rev	TGTTTCAGGTTTCAGGGGGAG	49.0
HRFRTF1	ACTGAAAAACCCCTGGCA	64.0
HRFRTF2	TGAAAAACCCCTGGCAGAC	65.0
HRFRTR1	CAACTGAAGGCGGACATTG	64.3
HRFRTR3	CCAAAACCAATCACCACCC	65.0

**Table 2.6**-Primers used in this study for amplification and sequencing. Large bold type indicates restriction sites added to primers to facilitate subcloning. 'Seq' denotes a sequencing primer.

#### 2.4.11.1 Primer Design

Primers used in this study were designed with the help of MacVector 7.2.2 (Oxford Molecular) software purchased from Invitrogen. The T<sub>m</sub> of primers was calculated using MacVector software. The primers were ordered from Sigma-Aldrich (Dorset, UK). A description of the primers used for PCR and sequencing can be found in Table 2.6.

#### 2.4.11.2 Cloning PCR Products

PCR products were cloned into vector pCR®2.1-TOPO® using the TOPO-TA cloning kit (Invitrogen) following manufacturer's instructions.

### 2.4.12 DNA Sequencing

Automated sequencing was carried out by the Geneservice Inc. (Cambridge, UK). Geneservice uses Applied Biosystems 3730 DNA Analyzer sequencers, which detect DNA molecules labelled with fluorescent dyes and automatically analyzes

data using Sun ultra10 sparcstations for sequence analysis. Primers used for sequencing can be found in Table 2.6.

## **2.5 Behavioural Analysis**

### **2.5.1 Equipment**

#### **2.5.1.1 Chambers**

Prior to behavioural recordings, flies were introduced into the appropriate chamber by aspiration through a small opening in the side of the chamber. For activity measurements, courtship behaviour and song recordings, round chambers 1 cm in diameter and 0.4 cm in height with a nylon mesh base were used. The chamber had a Perspex top to allow the camera to record the movements and activities of the flies.

#### **2.5.1.2 Camera**

A Sony DCR VX2100E camera was used for all behavioural recordings. The camera was mounted on an adjustable mount, and prepared for the recording by turning the power dial to 'Camera'. The camera was then positioned over the recording chamber, and the image on the LCD screen was sharpened using the focus and zoom rings. The audio input cable was connected to the MIC jack, and the input was switched to LINE. Sony Mini-DV (60 min SP) tapes were used in the camera. During the recording, each tape was named, and the date, genotype, time recording started, time recording ended, comments on recording, and eventual file name were noted on a logging sheet.

#### **2.5.1.3 INSECTAVOX**

An INSECTAVOX-like device was used to record the courtship song of *Drosophila melanogaster* males (Dryden and Kyriacou, unpub.; similar to the device described in Gorczyca and Hall, 1987). Briefly, the enclosure consists of three separate compartments: one containing a modified electret condenser microphone, a second containing the electronics (attenuator, pre-amplifier circuit, and a capacitor), and a third containing a low noise power supply and a transformer. The microphone is contained in an acoustically insulated

compartment to minimize the environmental noise on the recording. For recordings, the courtship chamber was placed directly onto the microphone, and the flies were viewed through a glass lens in the top of the INSECTAVOX-like device.

## **2.5.2 Computing**

### **2.5.2.1 Computer**

All computing for behavioural analysis was performed on a Power Mac G5 (Apple).

### **2.5.2.2 Digitizing and Compressing**

#### **Digitizing**

Behavioural recordings were digitized and compressed using FootTrack (v2.3.2) software (T-Squared Software). To digitize the tapes, the camera was connected to the G5 by a firewire cable. The tapes were then rewound, and to start digitizing, FootTrack was opened, and the 'New Tape' command under the 'File' menu was selected. The tape was given a name, and the type of tape selected was Hi8, with a 60 min recording time. Next, the 'Import' command was selected, and the 'OK' button was clicked. The digitized files were stored by FootTrack as .dv extensions.

#### **Compressing**

Digitized files were compressed using FootTrack software. Files to be compressed were highlighted and the 'Compress' option was selected. The type of compression selected was H.164. The compressed files were stored by FootTrack as .mov extensions.

#### **File Naming**

Files stored in FootTrack as .mov extensions were given names corresponding to the file names given on the initial behaviour logging sheet. Each tape was named by highlighting the file by clicking on the snapshot and pressing the 'i' button.

### **2.5.2.3 LifeSong X**

The LifeSong X program was obtained from J. Hall, A. Villella and J. Rieffel at Brandeis University (Villella *et al.*, 2005).

## **2.5.3 Behaviours**

### **2.5.3.1 Activity**

Activity was measured in the chambers described in section 2.5.1.1. 5 min recordings of single males were made, and the number of times the fly crossed a line across the middle of the chamber during the recording were counted (as described by Villella *et al.*, 1997). Activity was measured as the number of line crossings per minute at 25°C.

### **2.5.3.2 Courtship Behaviour**

Courtship behaviour was measured in the chambers described above, in a temperature controlled room set at 25°C; all courtship was recorded between 5 and 7 pm, prior to lights off at 8 pm. A single ‘courter’ (of varying genotype) and a single wild-type Canton S virgin ‘target’ female (1-day-old) were introduced into the chamber and observed for 5-10 min. Although experiments were performed in a temperature controlled room, the temperature within the INSECTAVOX-like device can increase slightly over the course of the testing interval. Therefore, to eliminate variation between genotypes tested in blocks at the beginning and the end of the interval, the recording sequence of individuals from different genotypes was randomized within the interval.

Courtship index (CI) was measured as the proportion of time in 5 min that the ‘courter’ spent courting the ‘target’ female (as described by Villella *et al.*, 1997). Wing extension index (WEI) was measured as the proportion of time in 5 min that the ‘courter’ spent extending its wing towards the ‘target’ female (as described by Villella *et al.*, 1997). Song index (SI) was determined to be the proportion of time spent by the ‘courter’ singing courtship song during wing extension.

### 2.5.3.3 Courtship Song

Several courtship song parameters were calculated, including the number of pulse trains per minute (PTPM) (as per Villella *et al.*, 1997), the number of sine song bouts per minute (SBPM) (as per von Schilcher, 1976c), the mean number of pulses per train (MPPT) (as per Villella *et al.* 1997), the number of cycles per pulse (CPP) (Bennet-Clark and Ewing, 1970; von Schilcher, 1976b; von Schilcher, 1977), and the interpulse interval (IPI) at 25°C (Ewing and Bennet-Clark, 1968). PTPM was calculated as (total number of pulses in 5 min recording/time in secs of recording) x 60. SBPM was calculated as (total number of bouts of sine song in 5 min/time in secs of recording) x 60. MPPT was calculated as the average number of pulses per train from all pulse trains where number of pulses was >2. CPP was calculated as the number of peaks per pulse from the first 10 pulse trains where pulses/train >10. IPI was calculated as the mean time between consecutive pulses in a pulse train, and was calculated from all pulse trains where the number of pulses per train >2.

## 2.6 Statistics

All statistics were performed using JMP Software v6.0 (SAS Institute). MPPT and IPI for each genotype were calculated as the mean of *n* intramale means. CI, WEI, and SI values were transformed using the square root arcsine transformation to approximate normality. These transformed values were subjected to one-way ANOVA analysis. Untransformed values for PTPM, SBPM, MPPT, CPP, and IPI were subjected to one-way ANOVA. Normality was determined by the Shapiro-Wilk W Goodness of Fit Test.

### **3 Dsx and Fru<sup>M</sup> in Courtship Song Production**

### 3.1 Introduction

Courtship song in *Drosophila melanogaster* is performed exclusively by the male, and is a critical component of male courtship behaviour, as it both stimulates the 'target' female to become more receptive to copulation, and contains species-specific information to allow the female to recognize conspecific males (Ewing and Bennet-Clark, 1968; Bennet-Clark and Ewing, 1969; Bennet-Clark and Ewing, 1970; von Schilcher, 1976a; von Schilcher, 1976c; Kyriacou and Hall, 1980; Kyriacou and Hall, 1982; Kyriacou and Hall, 1984; Rybak *et al.*, 2002). Like other courtship behaviours, song production has a genetic basis, and several genes such as *cacophony* (*cac*), *dissonance* (*diss*), *no-on-transient-A* (*nonA*), *period* (*per*), *transformer* (*tra*), *fruitless* (*fru*) and *doublesex* (*dsx*), have been identified which affect the quality of song production in males (von Schilcher, 1976b; von Schilcher, 1977; Kulkarni and Hall, 1987; Peixoto and Hall, 1998; Kulkarni *et al.*, 1988; Rendahl *et al.*, 1992; Rendahl and Hall, 1996; Kyriacou and Hall, 1980; Kyriacou and Hall, 1986; Kyriacou and Hall, 1989; Kyriacou *et al.*, 1990; Alt *et al.*, 1998; Bernstein *et al.* 1992; Ryner *et al.*, 1996; Villella *et al.*, 1997; Goodwin *et al.*, 2000; Villella and Hall, 1996; reviewed by Gleason, 2005).

Sex mosaic studies using male-female mosaics showed that the sex of the CNS is critical to the production of normal song (Hotta and Benzer, 1972; Hotta and Benzer, 1976; Hall, 1977; von Schilcher, 1977; von Schilcher and Hall, 1979), yet the precise contributions of sex determination genes to the specification of song production are unknown. Several sex determination genes such as *tra*, *fru* and *dsx* have been shown to affect song production (Kyriacou and Hall, 1980; Kulkarni *et al.*, 1988; Bernstein *et al.* 1992; Ryner *et al.*, 1996; Villella *et al.*, 1997; Goodwin *et al.*, 2000; Villella and Hall, 1996); therefore, by examining the individual, and combined, contributions of these genes to song production, a greater understanding of how the potential for sex-specific behaviours are programmed the CNS will be gained. In this chapter, the courtship song of flies with novel *fru* alleles will be analyzed alongside new mutant combinations of *fru* and *dsx*, to elucidate the relative roles of these sex determination genes in specifying courtship song.

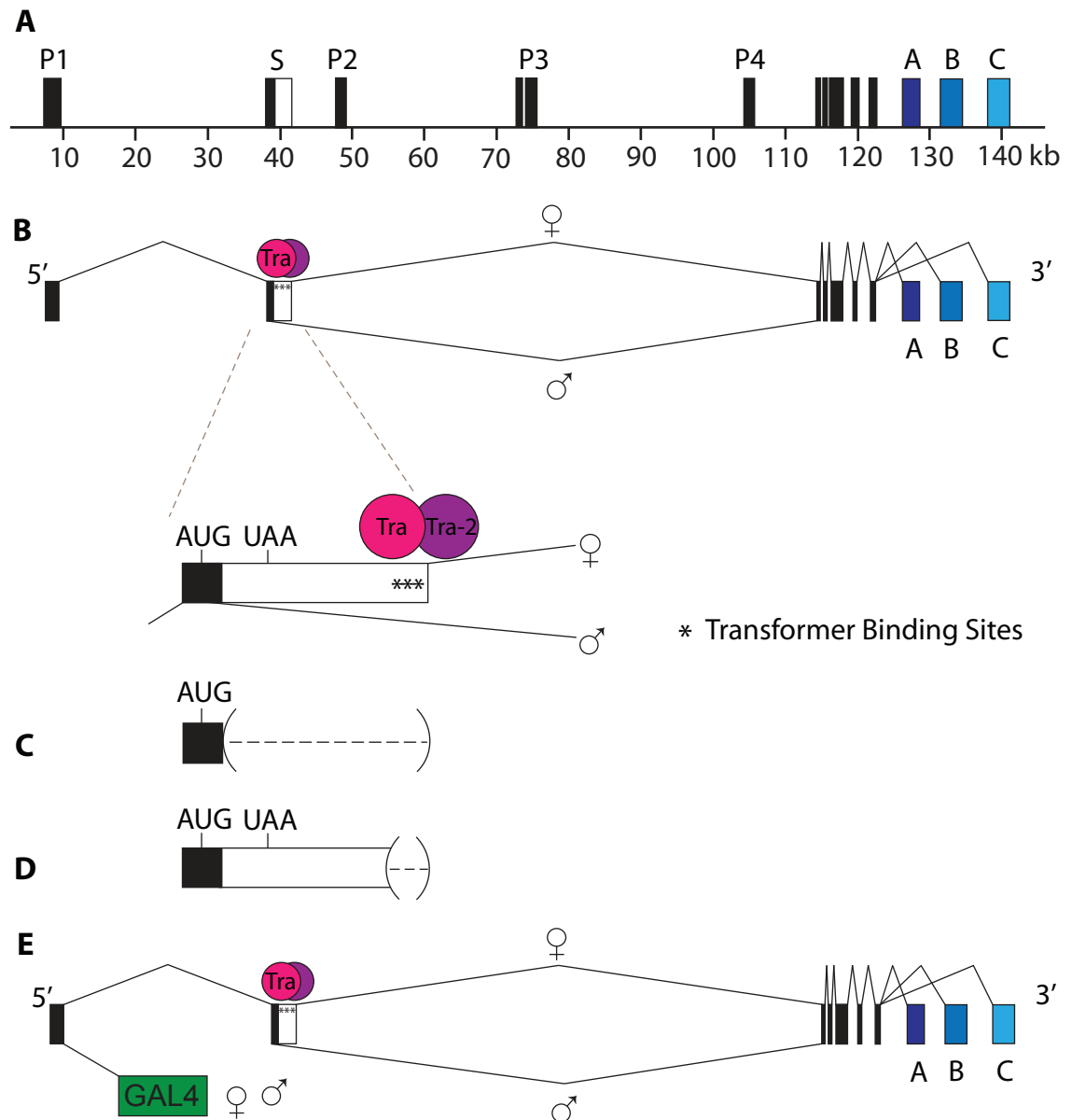
### 3.1.1 Song Production by *fru* and *dsx* Mutants

Courtship song consists of a low frequency humming sound called sine song, and a rhythmically patterned pulse song (von Schilcher, 1976c; Ewing and Bennet-Clark, 1968; Bennet-Clark and Ewing, 1970). Previous studies examined the individual roles of *fru* and *dsx* in song production by analyzing the quality of song in either *fru* or *dsx* mutant males, and found that *fru* mutants lack pulse song, whereas *dsx* mutants lack sine song (see Figure 1.5) (Villella and Hall, 1996; Ryner *et al.*, 1996; Villella *et al.*, 1997; Goodwin *et al.*, 2000). Thus *fru* and *dsx* are each required for courtship song. However, these studies did not determine the precise role of the male-specific isoforms of *fru*, called Fru<sup>M</sup> proteins, in courtship song production, nor did they address any potential interaction, or co-operation, between *fru* and *dsx* in the specification of song. Therefore, the relative contributions of both *fru* and *dsx* in specifying the sexually dimorphic production of courtship song will be investigated.

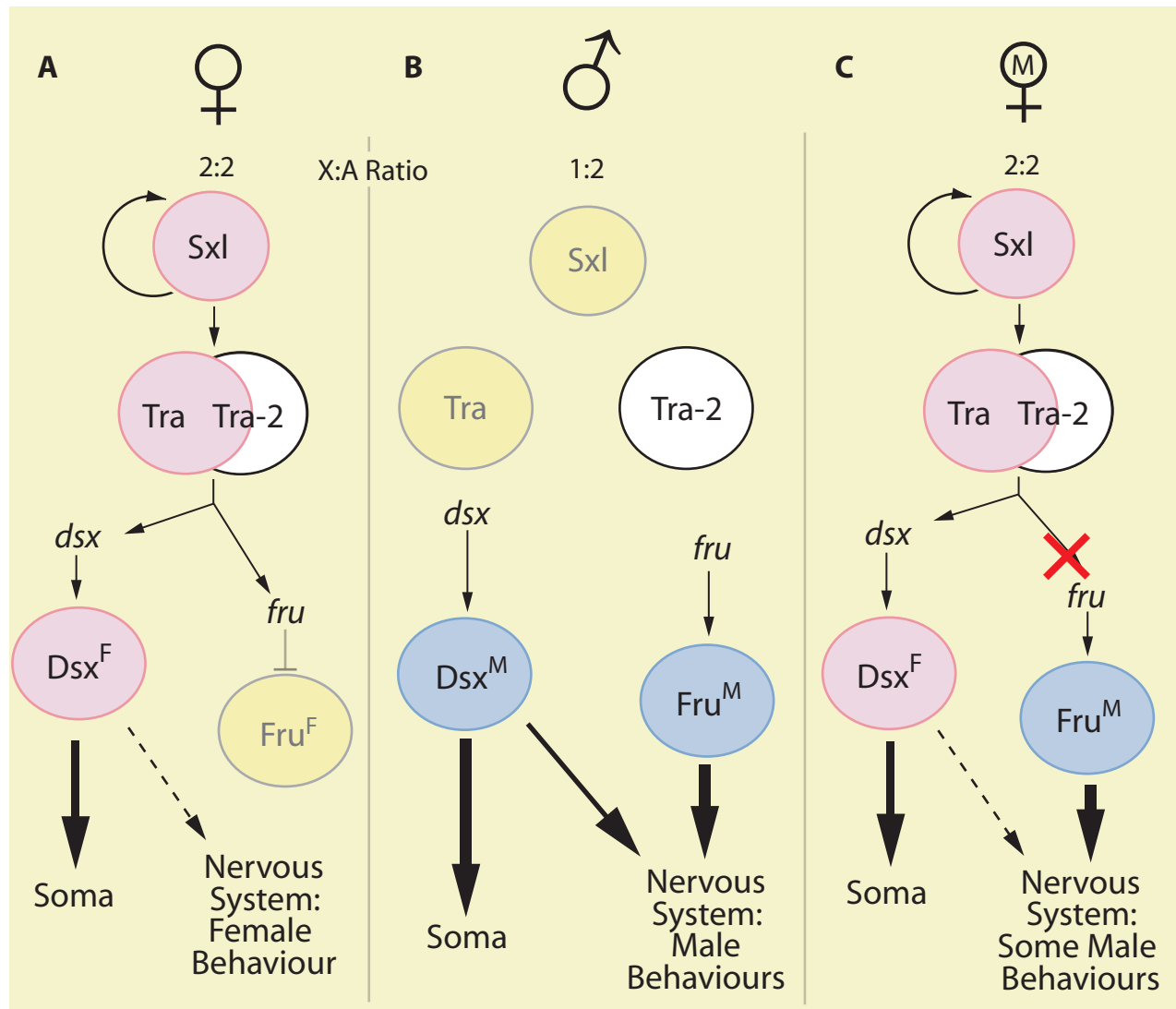
## 3.2 Song Production in Fru<sup>M</sup>-expressing Females

Recently, novel alleles of *fru*, *fru*<sup>M</sup> and *fru*<sup>Atra</sup>, were reported, in which females constitutively express the male-specific Fru<sup>M</sup> isoforms (Demir and Dickson, 2005). In wild-type females, transcripts from *fru*'s P1 promoter are spliced at the 5' end by the female-specific splice factor Transformer (Tra) and non-sex-specific Transformer-2 (Tra-2), which act together to cause a sex-specific splice in *fru* pre-mRNA near *cis*-acting repeat elements that are part of an exonic splicing enhancer called the transformer binding site (Figure 3.1) (Ryner *et al.*, 1996; Heinrichs *et al.*, 1998). The Tra-mediated female-specific splice introduces a stop codon into the transcript, and no functional Fru<sup>M</sup> proteins are translated (Ryner *et al.*, 1996). In *fru*<sup>M</sup> females, however, the female-specific portion of the S exon containing the Tra and Tra-2 binding site has been removed by homologous recombination, forcing the male-specific default splice, and resulting in the expression of Fru<sup>M</sup> proteins (Figures 3.1 and 3.2; Demir and Dickson, 2005). Similarly, *fru*<sup>Atra</sup> females also express Fru<sup>M</sup> proteins as a result of the deletion of only the Tra and Tra-2 binding site of the S exon, again by homologous recombination (Figures 3.1 and 3.2) (Demir and Dickson, 2005).





**Figure 3.1-** Alternative Splicing in *fru<sup>M</sup>* and *fru<sup>Δtra</sup>* Individuals. (A) Map of the *fru* locus indicating size of the gene, both the sex- and the non-sex-specific exons (solid-coloured boxes) and alternative promoters (P1-P4). Blue boxes indicate the alternative 3' end of the *fru* gene, zinc fingers A, B and C. (B) Alternative splicing in males and females in transcripts from *fru*'s P1 promoter at the S exon is shown. Male and female transcripts also undergo splicing at the 3' end, where each transcript will contain one of three zinc finger ends (A, B or C). Female-specific splice factor Tra, along with non-sex-specific Tra-2, cause a sex-specific splice in the *fru* transcript, and introduce a stop codon (UAA) into the female transcript. In males, a default splice occurs in the absence of Tra, and functional proteins, collectively called Fru<sup>M</sup> proteins, are translated. (C) *fru<sup>M</sup>* allele of *fru*, where the entire female-specific portion of the S exon has been deleted, forcing all transcripts in both males and females to undergo the male-specific splice. Fru<sup>M</sup> protein is translated in both sexes. (D) *fru<sup>Δtra</sup>* allele of *fru*, where only the Tra binding sites have been deleted from the S exon. Like the *fru<sup>M</sup>* allele, a male-specific splice is forced in both males and females, and Fru<sup>M</sup> protein is translated in both sexes. (E) *fru<sup>GAL4</sup>* allele, where the coding sequence for yeast transcriptional activator GAL4 is inserted into *fru*'s first intron, resulting in transcripts either containing GAL4, which will express GAL4 in *fru* neurons in both males and females, or in wild-type transcripts (if the GAL4 exon is bypassed), which will undergo sex-specific splicing as described in (B).



**Figure 3.2-** Sex Determination in *fru<sup>M</sup>* and *fru<sup>Atra</sup>* Females. (A) Sex determination pathway in a wild-type female. The ratio of 2 sex chromosomes to 2 autosomes results in the production of Sxl, which splices Tra into an active form, which then sex-specifically splices transcripts (along with Tra-2) from the *dsx* and *fru* genes, resulting in an individual with the female isoform of *dsx*, Dsx<sup>F</sup>, and a female-specific isoform of *fru*, which is not translated. (B) Sex determination pathway in a wild-type male. The ratio of one sex chromosome to two autosomes results in no Sxl being produced, and thus no Tra. Default splicing of *fru* and *dsx* genes occurs to give the male-specific isoforms of both *dsx* (Dsx<sup>M</sup>), and *fru* (Fru<sup>M</sup>) proteins. (C) Sex determination pathway in a *fru<sup>M</sup>* or *fru<sup>Atra</sup>* female. Sxl and Tra are present, as in wild-type females, and cause the sex-specific splicing of *dsx* transcripts to produce Dsx<sup>F</sup>. In the *fru* transcripts, the Tra binding sites have been deleted (see Figure 3.1), and result in the male-specific splicing of *fru* transcripts, and the production of Fru<sup>M</sup> isoforms in females. Adapted from Billeter *et al.*, (2006a).

The expression of  $Fru^M$  isoforms in the  $fru^M$  and  $fru^{Atra}$  females has been shown to induce the performance of most male behaviours (Demir and Dickson, 2005). However, these females show a subnormal levels of courtship behaviour compared to wild-type males, fail to attempt copulation, and it was not determined if they were able to perform courtship song. Thus, courtship behaviour, song production and quality of the  $fru^M$  and  $fru^{Atra}$  females were analyzed, to determine the precise role of  $Fru^M$  in specifying courtship song.

### **3.2.1 Courtship Behaviour of the $Fru^M$ -expressing Females**

#### **3.2.1.1 Courtship Index**

The courtship index (CI) is defined as the percentage of time spent by a 'courter' in courting a 'target' during the course of a behavioural recording (reviewed by Hall, 1994). A mean CI is then calculated for a given genotype, and compared to the CI of wild-type and control 'courtters'. Previously, it was reported that the  $fru^M$  and  $fru^{Atra}$  females had a CI of 30-40 (Demir and Dickson, 2005); only half of the CI of 85-90 observed in wild-type and control males. In this study, CI was recorded prior to analyzing courtship song to ensure that the results of the previous study could be replicated.

In this study, the CI of all  $fru^M$  and  $fru^{Atra}$  females tested (n=61) was found to fall within the range previously reported by Demir and Dickson (2005); however, the CIs of these females were not normally distributed ( $p < 0.05$ ; Shapiro-Wilk W Goodness of Fit Test), and therefore failed to meet a basic requirement for the use of parametric statistical methods. An examination of the CI values revealed that the  $fru^M$  and  $fru^{Atra}$  females fell into three distinct phenotypic classes: Class 1 females performed virtually no courtship, with a  $CI < 10\%$  (n=7); Class 2 females displayed orienting and following behaviours ( $CI > 10\%$ ), but never progressed to the later courtship steps of tapping, wing extension, courtship song and licking (n=25); Class 3 females performed all steps of the courtship ritual except attempted copulation (n=29). Only the 29 Class 3  $fru^M$  and  $fru^{Atra}$  females displayed wing extension and attempted courtship song, thus all song analysis was necessarily based upon these females. In addition, the CIs for the Class 3 females were

normally distributed ( $p > 0.05$ ; Shapiro-Wilk  $W$  Goodness of Fit Test), validating the use of parametric statistical tests to analyze the behaviour of these  $fru^M$  and  $fru^{Atra}$  females.

Table 3.1 shows the CIs of the 29  $fru^M$  and  $fru^{Atra}$  females. The mean CIs for  $fru^M$  ( $n=16$ ) and  $fru^{Atra}$  females ( $n=13$ ) were  $77.3 \pm 11.1$  and  $71.6 \pm 12.3$ , respectively, compared with CIs of  $92.7 \pm 6.7$  for wild-type CS males ( $n=15$ ), and  $95.2 \pm 2.7$  and  $95.4 \pm 2.9$  for control  $fru^M$  ( $n=15$ ) and  $fru^{Atra}$  males ( $n=18$ ). Although the CIs of the  $fru^M$  and  $fru^{Atra}$  females were significantly lower than the CIs of wild-type and control males ( $p < 0.05$ ), these findings demonstrate that some  $fru^M$  and  $fru^{Atra}$  females were capable of courting at nearly wild-type levels. Previous reports could be interpreted to suggest that  $fru^M$  and  $fru^{Atra}$  females can only court at levels approximately 50% of wild-type males (Demir and Dickson, 2005); however, results in this study suggest that the reported CI of 30-40 was a product of some females performing virtually no courtship, and others courting at almost wild-type levels.

Thus in describing the aberrant courtship behaviour of the  $fru^M$  and  $fru^{Atra}$  females, the main differences in the courtship behaviour of the  $fru^M$  and  $fru^{Atra}$  females, as compared to wild-type and control males, consisted of the reduced levels of courtship performed by the  $fru^M$  and  $fru^{Atra}$  females, and the high degree of variability in CI between females.

Genotype	<i>n</i>	CI	WEI	SI
Canton S (XY)	15	$92.8 \pm 6.8$	$38.3 \pm 2.8$	$87.9 \pm 2.4$
XY;; $fru^M / Df(3R)fru^{4-40}$	15	$95.3 \pm 2.7$	$53.0 \pm 2.9$	$90.3 \pm 2.0$
XY;; $fru^{Atra} / Df(3R)fru^{4-40}$	18	$95.4 \pm 3.0$	$54.6 \pm 2.8$	$85.3 \pm 2.2$
XX;; $fru^M / Df(3R)fru^{4-40}$	16	$77.1 \pm 11.1^*$	$37.6 \pm 3.3$	$44.8 \pm 4.7^*$
XX;; $fru^{Atra} / Df(3R)fru^{4-40}$	13	$71.7 \pm 12.3^*$	$31.4 \pm 2.9$	$60.1 \pm 5.6^*$
XX;; $tra^1 / Df(3L)st-J7$	10	$96.1 \pm 1.1$	$46.3 \pm 4.5$	$89.7 \pm 2.9$
XY;; $ln(3R)dsx^{23}, fru^3 / Df(3R)dsx^{15}, fru^3$	11	0*	0*	0*

**Table 3.1**-Behavioural analysis of  $fru^M$  and  $fru^{Atra}$  females. Behavioural parameters measured include CI, WEI, and SI. Values are shown as a mean of  $n \pm$  standard error of the mean (s.e.m.), where  $n$  is the number of individuals. \* indicates a significant difference from wild-type CS males.

### 3.2.1.2 Wing Extension Index

The wing extension index (WEI) is calculated as the percentage of time during a courtship recording that a courter extends its wing towards the target (as per Villella *et al.*, 1997). As only the Class 3 females performed wing extension, only these 29 *fru*<sup>M</sup> and *fru*<sup>Atra</sup> females were included in the analysis.

Interestingly, although the overall CI of the *fru*<sup>M</sup> and *fru*<sup>Atra</sup> females was significantly decreased compared to wild-type and control males, the WEI was not significantly different ( $p < 0.05$ ; Table 3.1; Appendix 1). The WEI of *fru*<sup>M</sup> females was  $37.6 \pm 3.3$  ( $n=16$ ), and the WEI of *fru*<sup>Atra</sup> females was  $31.4 \pm 2.9$  ( $n=13$ ), compared to  $38.3 \pm 2.8$  for wild-type males. In addition, the qualitative aspects of wing extension were normal, that is, the *fru*<sup>M</sup> and *fru*<sup>Atra</sup> females were able to extend their wings to an angle of  $90^\circ$  from their body, and would extend only the wing closest to the target individual.

Superficially, these results are in agreement with previous findings by Demir and Dickson (2005), who reported that the WEI of *fru*<sup>M</sup> and *fru*<sup>Atra</sup> females was not significantly different from wild-type and control males. However, the analysis in this study was performed exclusively on Class 3 females, who were capable of near wild-type levels of courtship, whereas the previous study presumably included data from all *fru*<sup>M</sup> and *fru*<sup>Atra</sup> females (Demir and Dickson, 2005). Given that in the previous study the CI was found to be 30-40, it is surprising that the females in the previous study were able to display wild-type levels of wing extension.

Thus, Class 3 *fru*<sup>M</sup> and *fru*<sup>Atra</sup> females are capable of qualitatively and quantitatively normal levels of wing extension; allowing the courtship song production of these females to be analyzed, and the contribution of *Fru*<sup>M</sup> to the specification of courtship song to be examined.

### 3.2.1.3 Song Index

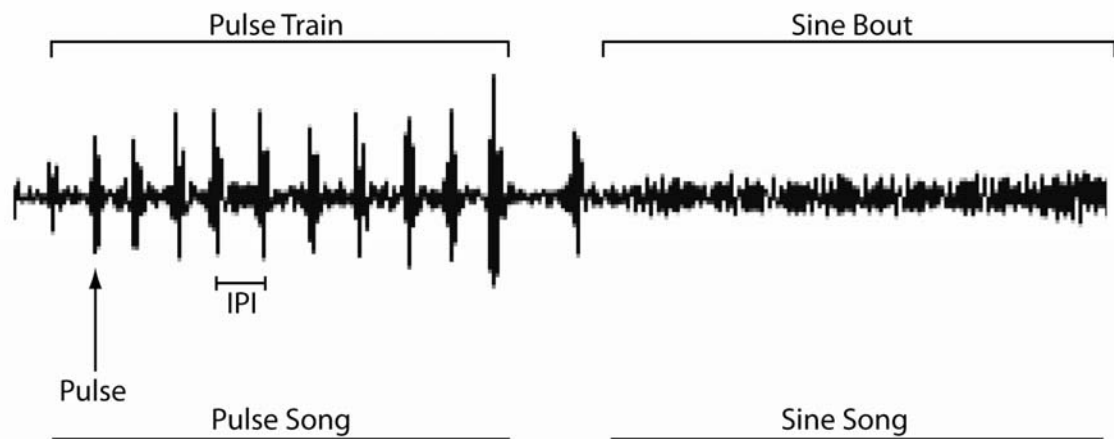
The song index (SI) was defined as the percentage of time spent singing during wing extension. In wild-type males, courtship song virtually always

occurs during wing extension, and the SI is an unnecessary measurement. However, the SI was included in the analysis of the *fru<sup>M</sup>* and *fru<sup>Atra</sup>* females, as it was not known prior to the analysis how much wing extension these females would perform. The SI was therefore used to distinguish between a defect in wing extension, and a defect in song initiation. For example, if the *fru<sup>M</sup>* and *fru<sup>Atra</sup>* females showed a significantly decreased amount of wing extension, but always attempted song production when the wing was extended, the SI would be normal, and it would suggest that the defect was not necessarily in song production, but in the process of extending the wing. On the other hand, if wing extension is normal, but the SI is very low, it suggests that there is a problem in the initiation of song that is not linked to an inability to extend the wing.

When the SI for Class 3 *fru<sup>M</sup>* and *fru<sup>Atra</sup>* females was calculated, the SI was significantly decreased compared to wild-type and control males ( $p < 0.05$ ; Table 3.1; Appendix 1). *fru<sup>M</sup>* females had an SI of  $44.8 \pm 4.7$  ( $n=16$ ), and *fru<sup>Atra</sup>* females an SI of  $60.1 \pm 5.6$  ( $n=13$ ), whereas the SI of wild-type CS males was  $87.9 \pm 2.4$  ( $n=15$ ). Thus, although the *fru<sup>M</sup>* and *fru<sup>Atra</sup>* females are capable of wild-type amounts of wing extension, the amount of song produced during wing extension was significantly less than in wild-type and control males. As discussed above, a normal WEI and a low SI strongly suggest a defect in song production, rather than in wing extension. Therefore, various parameters of song production were measured in *fru<sup>M</sup>* and *fru<sup>Atra</sup>* females, to fully describe the aberrant song phenotype of these females.

### **3.2.2 Song Quality in *Fru<sup>M</sup>*-expressing Females**

Courtship song quality is measured by various parameters, including the number of bouts of sine or pulse song per unit time (SBPM and PTPM), the mean number of pulses per pulse train (MPPT), and the mean time between consecutive pulses in a pulse train (IPI) (Figure 3.3) (as per Villella and Hall, 1996). The courtship song quality of the 29 Class 3 *fru<sup>M</sup>* and *fru<sup>Atra</sup>* females was determined by measuring these song parameters, and comparing the values to those observed in wild-type and control males.



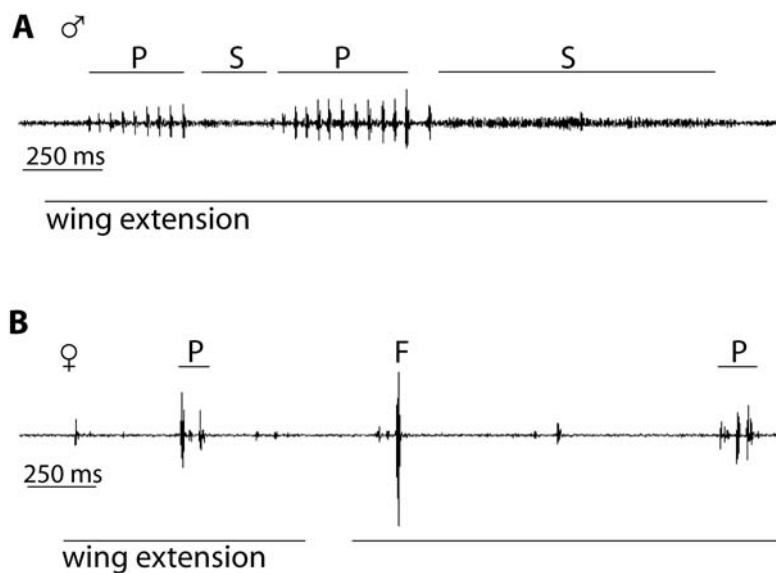
**Figure 3.3**-Song Parameters Included in Song Analysis. Representative song trace from a *Drosophila melanogaster* male, with both pulse song and sine song indicated below trace. Song parameters measured include: PTPM, where the pulse train is indicated above the trace; MPPT, where a pulse is indicated by an arrow below trace; IPI, shown below trace; and SBPM, where a bout of sine song is shown above the trace.

### 3.2.2.1 Pulse Song Production

Pulse song carries species-specific information, and allows the target female to identify conspecific males (Ewing and Bennet-Clark, 1968; Cowling and Burnet, 1981; Kawanishi and Watanabe, 1980; Kyriacou and Hall, 1982; Kyriacou and Hall, 1986). Wild-type CS males were found to perform  $19.9 \pm 1.4$  pulse trains per minute (PTPM) ( $n=15$ ), whereas *fru*<sup>M</sup> and *fru*<sup>Atra</sup> females produced significantly fewer PTPM, only  $7.1 \pm 1.5$  ( $n=16$ ) and  $9.4 \pm 1.4$  ( $n=13$ ) PTPM respectively ( $p<0.05$ ; Table 3.2; Appendix 1). Moreover, the mean number of pulses per train (MPPT) for the *fru*<sup>M</sup> and *fru*<sup>Atra</sup> females was also significantly decreased, where these females produced trains of only 3 pulses per train compared to  $8.1 \pm 0.3$  pulses per train for wild-type CS males ( $p<0.05$ ; Table 3.2). Wild-type males can produce pulse trains from 2 to 50 pulses in length (Kulkarni and Hall, 1987); however, the pulse trains of the *fru*<sup>M</sup> and *fru*<sup>Atra</sup> females were virtually never observed to exceed 3 pulses per train (Figure 3.4). Thus although the *fru*<sup>M</sup> and *fru*<sup>Atra</sup> females were able to extend their wings and attempt courtship song, they produced far fewer PTPM than wild-type males, and did not sustain a pulse train beyond 3 pulses.

Genotype	<i>n</i>	PTPM	MPPT	IPI (ms)	SBPM
Canton S (XY)	15	19.9 ± 1.4	8.1 ± 0.3	31.7 ± 3	18.6 ± 2.2
XY;; <i>fru<sup>M</sup>/Df(3R)fru<sup>A-40</sup></i>	15	28.5 ± 2.3	9.1 ± 0.3	32.0 ± 3	23.1 ± 1.9
XY;; <i>fru<sup>Atra</sup>/Df(3R)fru<sup>A-40</sup></i>	18	29.3 ± 1.8	9.6 ± 0.4	31.7 ± 4	20.6 ± 1.8
XX;; <i>fru<sup>M</sup>/Df(3R)fru<sup>A-40</sup></i>	16	7.1 ± 1.5*	3.0 ± 0.1*	26.5 ± 7*	0*
XX;; <i>fru<sup>Atra</sup>/Df(3R)fru<sup>A-40</sup></i>	13	9.4 ± 1.4*	2.9 ± 0.1*	24.3 ± 5*	0*
XX;; <i>tra<sup>1</sup>/Df(3L)st-J7</i>	10	19.2 ± 1.4	9.0 ± 0.2	33.0 ± 3	23.2 ± 1.8

**Table 3.2**-Song parameters of *fru<sup>M</sup>* and *fru<sup>Atra</sup>* females. Song parameters included are PTPM, MPPT, IPI and SBPM. Values shown for PTPM and SBPM are shown as the mean of *n* individuals ± standard deviation (s.d.). Values for MPPT and IPI are shown as the mean of *n* intramale means ± s.d. \* indicates a significant difference from wild-type CS males. Behavioural recordings were performed at 25°C. Raw behavioural data contained in Appendix 1.



**Figure 3.4**-Representative Song Trace from *fru<sup>Atra</sup>* Males and Females. A representative song trace of a fraction of the total recording time for *fru<sup>Atra</sup>* males (A) and *fru<sup>Atra</sup>* females (B) is shown, where wing extension during the recording is shown below the trace. Bouts of pulse song (P) and sine song (S) are indicated above the trace. 'F' indicates a noise caused by the target female falling during the recording.

The final parameter of pulse song measured was the IPI (Ewing and Bennet-Clark, 1968). The IPI is species-specific, and allows a female to identify a conspecific male (Ewing and Bennet-Clark, 1968; Kyriacou and Hall, 1982; Kyriacou and Hall, 1986; Ritchie *et al.*, 1999). Studies have shown that females prefer the IPI of their own species to that of other closely related species (Kyriacou and Hall, 1982). Interestingly, the length of the IPI has also been shown to cycle in a sinusoidal fashion, with a period of approximately



55-60 s (Kyriacou and Hall, 1980; Alt *et al.*, 1998). Songs containing this cycling have been shown to stimulate females to copulate more quickly than songs without cycling (Kyriacou and Hall, 1982; Ritchie *et al.*, 1999). Together, these results demonstrate the critical importance of the length of the IPI not only as a parameter of courtship song, but also to courtship success as a whole.

Wild-type CS males had an IPI of  $31.7 \pm 3$  ms ( $n=15$ ) at  $25^{\circ}\text{C}$ , which is in accordance with previously published IPI values for *Drosophila melanogaster* (Bennet-Clark, 1968; Kawanishi and Watanabe, 1980; Cowling and Burnet, 1981). The IPI of *fru*<sup>M</sup> females was  $26.5 \pm 7$  ms ( $n=16$ ), and the IPI in *fru*<sup>Atra</sup> females was  $24.3 \pm 5$  ms ( $n=13$ ), values significantly lower than those observed for wild-type and control males ( $p<0.05$ ; Table 3.2). In addition, given that the *fru*<sup>M</sup> and *fru*<sup>Atra</sup> females do not produce trains of more than 3 pulses, the cycling of the IPI could not be determined. Therefore, the quality of pulse song of the *fru*<sup>M</sup> and *fru*<sup>Atra</sup> females was extremely poor, with fewer pulse trains, fewer pulses per train, and an aberrant IPI.

### 3.2.2.2 Sine Song Production

Sine song is a low frequency humming noise, where the number of bouts per minute can be quantified (von Schilcher, 1976c; Vilella and Hall, 1996). Typically, wild-type males will alternate between pulse song and sine song during a given bout of courtship song (von Schilcher, 1976c). In this study, the SBPM performed by wild-type males was  $18.6 \pm 2.2$  bouts per minute ( $n=15$ ; Table 3.2), whereas the 29 Class 3 *fru*<sup>M</sup> and *fru*<sup>Atra</sup> females were never observed to produce any bouts of sine song. Thus in addition to the subnormal amounts of song produced by *fru*<sup>M</sup> and *fru*<sup>Atra</sup> females, various parameters of both pulse and sine song were also found to be highly aberrant, demonstrating that *Fru*<sup>M</sup> expression alone does not specify wild-type song production or quality.

### 3.2.3 *Fru*<sup>M</sup> Does Not Specify Courtship Song

A thorough examination of the courtship behaviour and courtship song production in *fru*<sup>M</sup> and *fru*<sup>Atra</sup> females clearly shows that *Fru*<sup>M</sup> expression is

not the sole determinant of male sexual behaviour in *Drosophila melanogaster*. Therefore, although Fru<sup>M</sup> can specify some aspects of male-specific behaviours, other factors must be involved in order for a full complement of male behaviours to be achieved.

### 3.3 Dsx<sup>M</sup> in Courtship Song Production

An obvious candidate to act alongside Fru<sup>M</sup> in the specification of courtship song is *dsx*. Although previous studies have discounted the role of *dsx* in the specification of male behaviours (Demir and Dickson, 2005), *dsx* mutant males display subnormal levels of courtship, and have an aberrant song phenotype (Taylor *et al.*, 1994; Villella and Hall, 1996). Significantly, *dsx* mutants lack sine song (Villella and Hall, 1996); a phenotype shared by *fru*<sup>M</sup> and *fru*<sup>Atra</sup> females. Thus the role of *dsx*, alongside *fru*, in the specification of courtship song was examined in further detail.

To dissect the individual contributions of *fru* and *dsx* to the specification of courtship song, the courtship behaviour and courtship song of males lacking both Fru<sup>M</sup> and Dsx (genotype *In(3R)dsx<sup>23</sup>, fru<sup>3</sup>/Df(3R)dsx<sup>15</sup>, fru<sup>3</sup>*) were examined. These double mutants had a CI of 0 towards females (n=11), and therefore produced no song (Table 3.1). This suggests that in the absence of Fru<sup>M</sup> and Dsx, courtship song is not produced.

It was shown in section 3.2 that Fru<sup>M</sup> expression alone in females was not sufficient for courtship song. Similarly, the expression of Dsx<sup>M</sup> in females is also not sufficient for song (Taylor *et al.*, 1994). Thus neither *fru* nor *dsx* alone can specify courtship song. In fact, only in *tra* mutant females, where both Fru<sup>M</sup> and Dsx<sup>M</sup> are present, are wild-type levels of courtship behaviour observed, a finding confirmed here (Tables 3.1 and 3.2) (Kyriacou and Hall, 1980; Kulkarni *et al.*, 1988; Bernstein *et al.*, 1992). Together, these results demonstrate a previously unrecognized requirement for Dsx<sup>M</sup> alongside Fru<sup>M</sup> in the generation of courtship song and a therefore a full complement of male behaviours.

### 3.4 Discussion

In this chapter, the relative contributions of *fru* and *dsx* to the specification of courtship song were examined, in order to gain insight into the genetic basis for the sexually dimorphic production of courtship song.

#### 3.4.1 *Fru<sup>M</sup>* and *Dsx<sup>M</sup>* Are Both Required for Male Behaviour

In the sex determination hierarchy, *Tra* is responsible for the sex-specific splicing of both *dsx* and *fru* transcripts (Nagoshi *et al.*, 1988; Burtis and Baker, 1989; Ryner and Baker, 1991; Hedley and Maniatis, 1991; Hoshijima *et al.*, 1991; Ryner *et al.*, 1996; Heinrichs *et al.*, 1998). Studies have shown that *dsx* determines the sex of the external cuticular structures and pheromones, as well as the sex of the internal reproductive anatomy and physiology (Hildreth, 1965; McRobert and Tompkins, 1985; Jallon *et al.*, 1988; Nagoshi *et al.*, 1988; Baker and Wolfner, 1988; Burtis and Baker, 1989; Jursnich and Burtis, 1993; Waterbury *et al.*, 1999). *fru*, on the other hand, is largely responsible for determining the sex of the CNS (Taylor *et al.*, 1994; Ryner *et al.*, 1996; Usui-Aoki *et al.*, 2000; Kimura *et al.*, 2005; Demir and Dickson, 2005). These findings led to the suggestion that *fru* and *dsx* sit atop independent branches of the sex determination pathway, where *dsx* controls the sex of non-neuronal tissues, and *fru* determines the sex of the CNS. Mounting evidence, however, suggests that *dsx* plays a larger role, alongside *fru*, in the determination of male sexual behaviour (Taylor and Truman, 1992; Villella and Hall, 1996; Waterbury *et al.*, 1999; Lee *et al.*, 2002; Fujii and Amrein, 2002; Dauwalder *et al.*, 2002; Bray and Amrein, 2003; Billeter *et al.*, 2006b; Lazareva *et al.*, 2007).

Demir and Dickson (2005) showed that *Fru<sup>M</sup>* expression in females triggered the performance of male-specific behaviours. Similar results were found when chromosomal females mutant for both *tra* and *dsx* were tested (who would express *Fru<sup>M</sup>* but no *Dsx*) (Taylor *et al.*, 1994). These studies suggest that *Fru<sup>M</sup>* expression alone is sufficient for the performance of some male-specific behaviours; however, a closer examination of the courtship behaviour of the *fru<sup>M</sup>* and *fru<sup>Δtra</sup>* females in this chapter, including a detailed study of wing extension and song production, showed that *Fru<sup>M</sup>* alone does

not specify all male courtship behaviours. Instead, a clear requirement for  $Dsx^M$ , alongside  $Fru^M$ , in the specification of a full complement of male behaviours was demonstrated.

### Courtship Behaviour

An examination of the courtship behaviour of  $fru^M$  and  $fru^{Atra}$  females revealed an enormous amount of variation between individuals of the same genotype. Some  $Fru^M$ -expressing females performed almost wild-type levels of courtship (though with aberrant song production), whereas others performed virtually no courtship whatsoever. This variability in behaviour was not linked to individual variation in  $Fru^M$  expression, as all females expressed  $Fru^M$  in their CNSs. Therefore, measuring an overall CI to describe the courtship behaviour of all  $Fru^M$ -expressing females examined ( $n=61$ ) was inappropriate in this study, as the data were not normally distributed. Thus the  $fru^M$  and  $fru^{Atra}$  females were subdivided into behavioural classes, and further behavioural analyses performed on a subset of these females ( $n=29$ ).

The inconsistency in behaviour of the  $fru^M$  and  $fru^{Atra}$  females not only applies to the amount of behaviour(s) shown by an individual during courtship, but also to the range of behaviours performed during an attempt at courtship. Of the females who attempted courtship ( $n=54$ ), only the 29  $fru^M$  and  $fru^{Atra}$  females who performed nearly wild-type levels of courtship displayed all of the steps in the courtship ritual (except attempted copulation); the other 25 females displayed only orientation and following. Thus the progression from the early stages (orienting and following) to the later stages of courtship (wing extension, song and licking) was not automatic in these females, suggesting that sensory cues or stimuli required for progression to occur are not being received and/or processed appropriately in the  $fru^M$  and  $fru^{Atra}$  females.

A variety of sensory inputs are required for efficient courtship, including olfactory, gustatory, visual, and auditory cues (reviewed by Tompkins, 1984; Hall, 1994; Billeter *et al.*, 2002; Billeter *et al.*, 2006a). Removing any one of these inputs results in decreased courtship (Gailey *et al.*, 1986; Joiner and Griffith, 1997; reviewed by Greenspan and Ferveur, 2000); however, only

when virtually all sensory cues are removed is courtship behaviour in wild-type males abolished (Gailey *et al.*, 1986), suggesting a great deal of redundancy in mate recognition sensory cues. The lack of progression to the later stages of the courtship ritual in many of the *fru<sup>M</sup>* and *fru<sup>Atra</sup>* females suggests a disruption in either the ability to receive certain required stimuli, or in the ability to process sensory cues to stimulate the appropriate behavioural response.

Visual stimuli are critical in the identification of a potential mate; constituting some of the first sensory cues received by the male during courtship (Connolly *et al.*, 1969; Tompkins *et al.*, 1982). Courtship can be triggered by female movement and shape (Connolly *et al.*, 1969; Tompkins *et al.*, 1982), and continues as the male ‘tracks’ the movements of the female (Cook, 1979; Cook, 1980). Males with visual impairments show an overall reduction in CI (Geer and Green, 1962; Grossfield, 1966; Connolly *et al.*, 1969; DeJianne *et al.*, 1981; Tompkins *et al.*, 1982; Markow, 1987; Chatterjee and Singh, 1988; Stocker and Gendre, 1989; Joiner and Griffith, 1997); however, they are still able to perform all steps of the courtship ritual (Gailey *et al.*, 1986; Joiner and Griffith, 1997). In larger flies, like *Sarcophaga bullata* and *Calliphora erythrocephala*, male-specific giant neurons in the lobula have been observed (Gilbert and Strausfeld, 1991; Gronenberg and Strausfeld, 1991; Strausfeld, 1991), suggesting that the visual systems of males and females may not be equivalent. Moreover, the lobula, an optic neuropil implicated in tracking behaviour during courtship (Strausfeld, 1980), has been reported to be slightly larger in male *Drosophila* than in females (Heisenberg *et al.*, 1995; Rein *et al.*, 2002). Thus, it is possible that the *fru<sup>M</sup>* and *fru<sup>Atra</sup>* females, who do not initiate any courtship, fail to visually identify appropriate targets of courtship. Alternatively, the females who do initiate courtship may not be capable of the sophisticated ‘tracking’ performed by male flies, to keep in close proximity to the target, a pre-requisite to receiving further sensory cues and stimulation, hastening progression to the later stages of courtship.

As with visual cues, pheromonal cues can also stimulate the male to perform courtship behaviour (Shorey and Bartell, 1970; Averhoff and Richardson 1974; Tompkins, 1984; Jallon, 1984; Markow, 1988; Coyne *et al.*, 1994).

Volatile female pheromones are detected by the olfactory system of the male prior to contact with the 'target' female (Shorey and Bartell, 1970; Averhoff and Richardson 1974; Tompkins, 1984; Coyne *et al.*, 1994; Ha and Smith, 2006; Kurtovic *et al.*, 2007); and later, during physical contact, olfactory and gustatory cues are exchanged between the sexes, primarily in the form of non-volatile pheromones (Jallon, 1984; Markow, 1987; Greenspan and Ferveur, 2000; Amrein and Thorne, 2005). Given that males and females emit sex-specific chemosensory cues (Jallon, 1984), it is unsurprising that dimorphisms have been identified within chemosensory structures in flies (Nayak and Singh, 1983; Possidente and Murphey, 1989; Kondoh *et al.*, 2003; Bray and Amrein, 2003; Stockinger *et al.*, 2005). For example, three glomeruli of the antennal lobe in *Drosophila melanogaster*, thought to be involved in pheromone reception, have been identified as being sexually dimorphic in volume (Kondoh *et al.*, 2003; Stockinger *et al.*, 2005). Moreover, this dimorphism in glomerular volume has been shown to be dependant on the expression of *fru*; the expression of Fru<sup>M</sup> in *fru*<sup>M</sup> and *fru*<sup>Atra</sup> females abolished the difference in volume between the sexes (Stockinger *et al.*, 2005). As such, the decreased amount of courtship observed in *fru*<sup>M</sup> and *fru*<sup>Atra</sup> females is therefore not likely a result of dimorphic glomerular volume. However, recent studies have found that both males and females express an olfactory receptor for the volatile pheromone 11-*cis*-vaccenyl acetate (cVA); the behavioural responses to the loss of this receptor in males and females are completely different (Ha and Smith, 2006; Kurtovic *et al.*, 2007). This result suggests that even though both sexes are able to receive the same pheromonal cue, differential processing in the CNS can lead to alternate behavioural responses. Therefore, the decreased levels of courtship in *fru*<sup>M</sup> and *fru*<sup>Atra</sup> females may be a result of a lack of olfactory stimulation, a result of inappropriate processing of olfactory cues.

Another relevant difference in the chemosensory system is the sexually dimorphic number of gustatory bristles on the foreleg; males have been found to have on average 50, whereas females generally have only 37 (Nayak and Singh, 1983; Possidente and Murphey, 1989; Meunier *et al.*, 2000). The neurons of ten of these male-specific taste bristles express the gustatory receptor gene *Gr68a* (Bray and Amrein, 2003), a putative taste receptor for the female-specific pheromones 7,11-nonacosadiene and 7,11-

heptacosadiene (Bray and Amrein, 2003). The lack of either *Gr68a*-expressing neurons, or *Gr68a* expression, allows normal (or even elevated) initiation of courtship; however, the overall CI and later stages of courtship (wing extension and attempted copulation) are significantly decreased (Bray and Amrein, 2003). This phenotype is similar to the aberrant courtship seen in *fru<sup>M</sup>* and *fru<sup>Atra</sup>* females, where an overall decrease in CI and specifically the later stages of courtship was observed. Intriguingly, the expression of *Gr68a* was found to be *dsx*-dependent, where the expression of *Dsx<sup>M</sup>* was sufficient to direct the expression of *Gr68a* in the male-specific foreleg bristles (Bray and Amrein, 2003). The probable lack of *Dsx<sup>M</sup>* (and *Gr68a*) in *fru<sup>M</sup>* and *fru<sup>Atra</sup>* females could, at least partially, explain why many females do not progress beyond the early stages of courtship, as they are not sufficiently stimulated by the requisite gustatory cues. Moreover, the dependence of *Gr68a* on *Dsx<sup>M</sup>* could, again at least partially, explain the requirement for *Dsx<sup>M</sup>* alongside *Fru<sup>M</sup>* in the specification of a full complement of male courtship behaviours.

A final explanation for the lower levels of courtship performed by the *fru<sup>M</sup>* and *fru<sup>Atra</sup>* females relates to the role of the fat body in modulating courtship behaviour. It has been shown that the sex of the adult fat body is critical to the performance of wild-type levels of courtship, where the feminization of the fat body leads to a significant decrease in courtship behaviour in males (Fujii and Amrein, 2002; Dauwalder *et al.*, 2002; Lazareva *et al.*, 2007). Recently, Lazareva *et al.* (2007) extended these findings, and showed that the courtship behaviour of the *fru<sup>M</sup>* and *fru<sup>Atra</sup>* females could be significantly improved if the fat body in these females was genetically ‘masculinized’. Thus the poor courtship behaviour performed by the *fru<sup>M</sup>* and *fru<sup>Atra</sup>* females can also be attributed to the presence of a ‘female’ fat body.

### **3.4.2 What is the Aetiology of the Song Defect in *Fru<sup>M</sup>*-expressing Females?**

In addition to the aberrant and inconsistent courtship behaviour(s) displayed by the *fru<sup>M</sup>* and *fru<sup>Atra</sup>* females, the low levels and poor quality of the song produced compared to wild-type or control males are striking. How might

these differences arise? Given the importance of the sex of the CNS to song production (Hall, 1979; von Schilcher and Hall, 1979), it seems likely that the relevant differences will lie within the CNS, either in sexually dimorphic motor output, or in the sex-specific neural circuits which control the motor output.

A clear requirement for both *dsx* and *fru* in the specification of courtship song has been demonstrated; however, it is not clear how these two genes create the potential for this sexually dimorphic behaviour. *Fru<sup>M</sup>* has been shown to create sex-specific axonal morphology and neuron numbers (Billeter and Goodwin, 2004; Kimura *et al.*, 2005), whereas *Dsx<sup>M</sup>* is known to be involved in creating sexually dimorphic neuron numbers in specific regions of the CNS (Taylor and Truman, 1992). Thus *Fru<sup>M</sup>* and *Dsx<sup>M</sup>* may create both a sexually dimorphic motor output, and a sex-specific neural circuitry to govern the motor output which controls courtship song production, possibilities that will be examined in the coming chapters.

Overall, the investigation of the relative contributions of *fru* and *dsx* to courtship song production revealed an unrecognized requirement for both *Fru<sup>M</sup>* and *Dsx<sup>M</sup>*. Significantly, it is the first demonstration of the co-operation of the two in the specification of a male-specific behaviour.

### **3.4.3 Conclusions**

In this chapter, the relative contributions of *fru* and *dsx* to the specification of some courtship behaviours and song were investigated.

Previous reports showed that *fru<sup>M</sup>* and *fru<sup>Atra</sup>* females perform many steps of the male-specific courtship behaviour, and as a result, it was suggested that *fru* is a 'genetic switch' for male courtship behaviours (Demir and Dickson, 2005). However, a more detailed examination of the courtship behaviour of the *fru<sup>M</sup>* and *fru<sup>Atra</sup>* females revealed a high degree of variation in the amount of courtship behaviour(s) observed in these females. In addition, many *fru<sup>M</sup>* and *fru<sup>Atra</sup>* females failed to progress beyond the early stages of courtship, suggesting a lack of stimulation by sensory cues, either as a result



of an inability to receive the cues, or a defect in processing the cues to produce an appropriate behavioural response.

Of the *fru*<sup>M</sup> and *fru*<sup>Δtra</sup> females who attempted courtship song, the amount of courtship song was significantly decreased and the song quality was extremely poor. Only when both *Dsx*<sup>M</sup> and *Fru*<sup>M</sup> were expressed could wild-type levels and quality of song be achieved, demonstrating a novel requirement for *Dsx*<sup>M</sup> alongside *Fru*<sup>M</sup> in the specification of courtship song.

Although a requirement for both *Fru*<sup>M</sup> and *Dsx*<sup>M</sup> in the specification of courtship song was shown, it remains unclear what *fru*- or *dsx*-regulated dimorphisms underlie this sex-specific production of courtship song. Given that the sex of the CNS is central to the production of courtship song (von Schilcher and Hall, 1979), it is likely that the relevant differences arise as a result of either a sexually dimorphic motor output, or from sex-specific neural circuitry controlling song production. The ensuing chapters will therefore investigate the neurobiological basis for the sex-specific production of courtship song, and dissect the relative roles of *fru* and *dsx* in creating these differences.

## **4 Characterization of the Motor Neurons Innervating the Direct Flight Muscles**

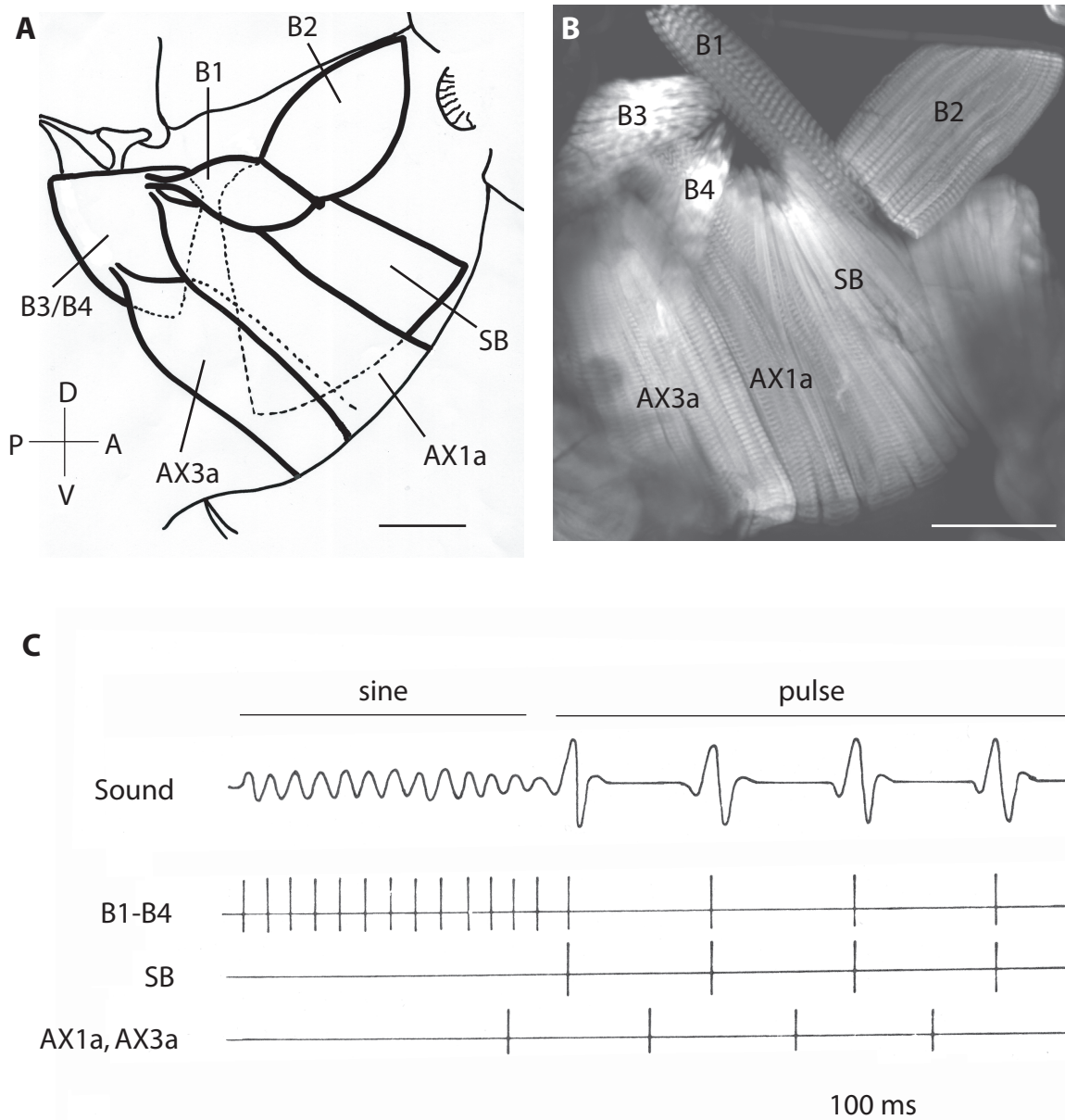
## 4.1 Introduction

Courtship song is generated when the male fly extends and vibrates one of his wings. A number of studies have described the anatomical, neurobiological and electrophysiological properties of the structures involved in the production of courtship song in males; however, equivalent experiments were not performed in both sexes. Thus, the underlying basis for the sexually dimorphic production of courtship song is unknown.

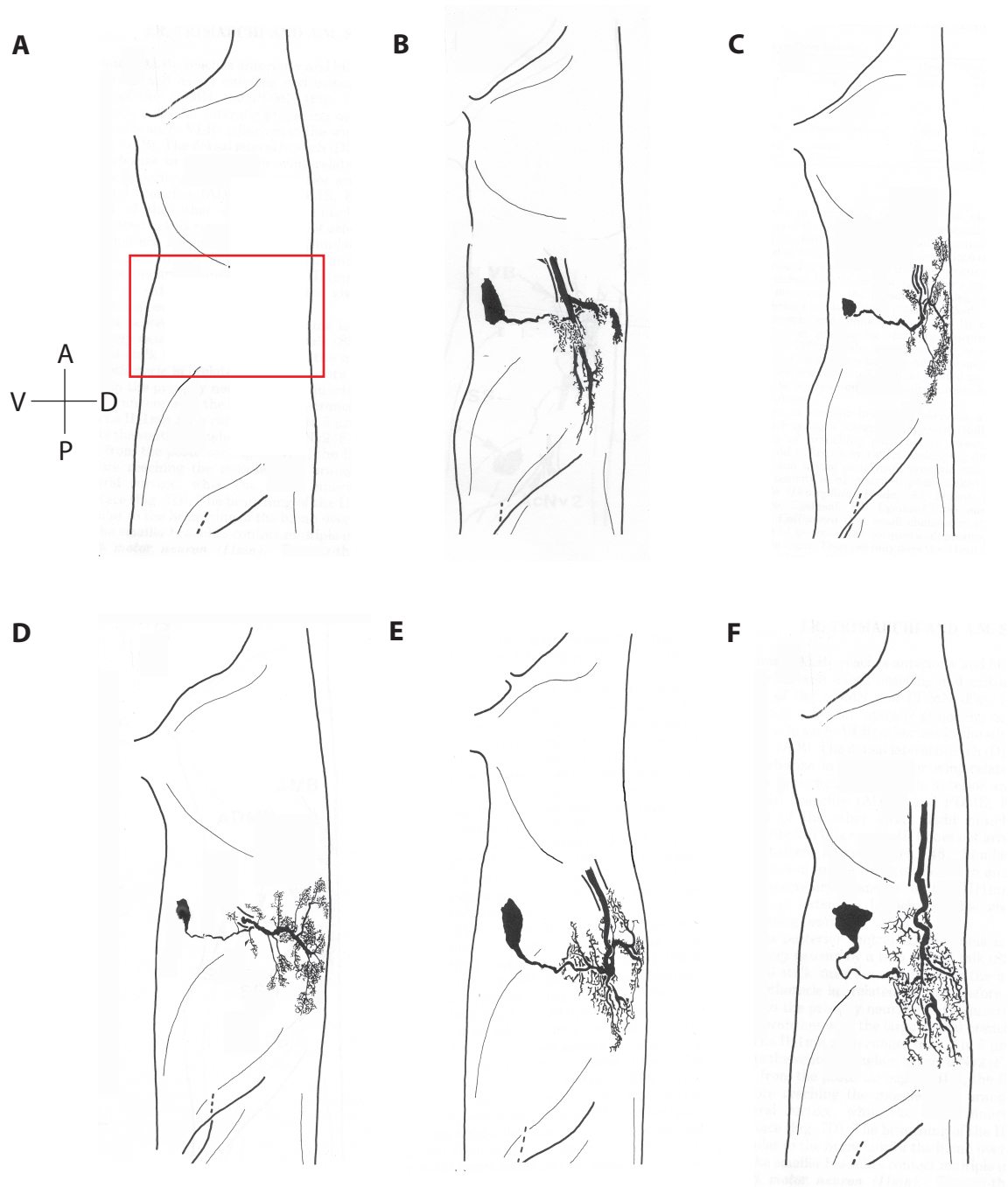
In the previous chapter, it was shown that two sex determination genes, *fru* and *dsx*, are both required for the specification of courtship song, however, no *fru*- and/or *dsx*-regulated structures underlying the sexually dimorphic production of courtship song have been reported. Studies using male-female mosaics have shown that the sex of the CNS is directly related to the ability to produce wild-type courtship song (Hall, 1979; von Schilcher and Hall, 1979). Indeed, provided that specific regions of the CNS are male, an individual with a female body is capable of producing normal courtship sounds (von Schilcher and Hall, 1979). Therefore, the sexually dimorphic production of courtship song can be attributed either to the sexually dimorphic motor output of the CNS, the presence of sex-specific neural circuitry in the CNS governing the sexually dimorphic output, or some combination of both. Therefore, to determine whether the sexually dimorphic production of courtship song is a result of sex-specific motor output, this chapter will document and compare the neurobiological properties of the motor neurons responsible for courtship song production in males and females.

### 4.1.1 Direct Flight Muscles

In *Drosophila*, there are ten main direct and axillary flight muscles (DFMs) per hemithorax, located around the wing hinge (Ewing, 1979). The DFMs develop *de novo* during metamorphosis from the fusion of myoblasts (Lawrence, 1982; Ghazi *et al.*, 2000; Sudarsan *et al.*, 2001; Kozopas and Nusse, 2002). The primary roles of the DFMs in both males and females are to extend and fold the wing, and to steer the wing during flight (Ewing, 1977; Ewing, 1979; Miyan and Ewing, 1985). In males, the DFMs have an additional role in courtship song production (Ewing, 1979), where the electrophysiological activity of the DFMs has been directly related to the beating of the wing during song (Figure 4.1; Ewing, 1979).



**Figure 4.1**-Activity of the Direct Flight Muscles During Courtship Song. (A) Schematic representation of the DFMs. The basalar muscles (B1-B4), anterior muscles of the first and third axillary (AX1a, AX3a), and the sternobasalar muscle (SB) are indicated (adapted from Trimarchi and Schneiderman, 1994, nomenclature as per Ewing, 1979). Anterior, posterior, ventral and dorsal positions are indicated. (B) Maximum Z-projection of a confocal image of the DFMs, visualized by counterstaining with TRITC-conjugated phalloidin. B1-B4, AX1a, AX3a, and SB are indicated. (C) Song trace of a wild-type male; pulse song and sine song are indicated above trace. Action potentials are shown for DFMs B1-B4, SB, and AX1a, AX3a below the song trace (from Ewing, 1979). B1-B4 fire once per wing beat during both sine song and pulse song. SB, on the other hand, is silent during sine song, but fires once per wing beat during pulse song. AX1a and AX3a are also silent during sine song, but are active during pulse song, firing approximately 15 ms before an individual sound pulse. Scale bars = 100  $\mu$ m.



**Figure 4.2**-Cell bodies of the mnDFM lie in the Msg (from Trimarchi and Schneiderman, 1994). Camera Lucida drawings of the cell body positions of the mnDFMs within the Msg. (A) Schematic representation of a lateral view of the VNC of the CNS. Anterior, posterior, ventral and dorsal positions are indicated. Red box outlines the Msg. Cell bodies and dendritic projections of (B) mnB2, (C) vmnB3/B4, (D) dmnb3/B4, (E) mnAX1a, (F) mnSB (all nomenclature as per Ewing, 1979) are shown, all of which project dorsally from a ventrally-positioned cell body.

Seven of the DFMs from which the most consistent electrophysiological recordings were obtained, and whose activity bears the most direct relationship to the beating of the wing during song, are the four basalar muscles (B1-B4), the anterior muscles of the first and third axillary (AX1a and AX3a), and the sternobasalar muscle (SB) (nomenclature as per Ewing, 1979) (Figure 4.1). Both males and females have a full complement of DFMs for flight, making it unlikely that the courtship song is due to a dimorphism within the musculature itself. In addition, von Schilcher and Hall (1979) showed that provided that specific regions of the CNS were male, a female wing (and associated musculature) was able to produce wild-type courtship song, suggesting that the sex of the CNS controls the quality of the courtship song. Therefore, critical differences leading to the sex-specific production of song may lie in the modulation of the activity of the DFMs by their associated motor neurons. Indeed, the DFMs are neurogenic (Ewing, 1977), and require action potentials for each wing beat, suggesting that any sex-specific properties of these motor neurons would greatly impact motor output, and hence courtship song. Therefore, an investigation of the motor neurons innervating seven of the DFMs was performed, to document any sex-specific properties of these neurons which could be responsible for the male-specific production of courtship song.

#### ***4.1.2 Motor Neurons Innervating the Direct Flight Muscles (mnDFMs)***

The axonal and dendritic morphologies of the motor neurons innervating six of the seven DFMs were previously reported (Trimarchi and Schneiderman, 1994). In the previous study, the mnDFMs were backfilled with HRP, and Nomarski optical interference was used to visualize the motor neurons on the muscles (Trimarchi and Schneiderman, 1994). Four of the DFMs, B2, AX1a, AX3a, SB were reported to be innervated by single motor neurons called mnB2, mnAX1a, mnAX3a, and mnSB, respectively. Two motor neurons, on the other hand, called vmnB3/B4 and dmnB3/B4, were each found to innervate both the B3 and B4 muscles (Trimarchi and Schneiderman, 1994). Significantly, Trimarchi and Schneiderman (1994) also reported the cell body locations of the mnDFMs (Figure 4.2), and found the cell bodies of most mnDFMs lie in the ventral thoracic ganglia of the CNS (Trimarchi and Schneiderman, 1994). This result is very interesting, given that sex mosaic studies have shown that the neural foci for the sexually

dimorphic production of courtship song likely lie in the ventral thoracic ganglia of the CNS (von Schilcher and Hall, 1979). Therefore, in this study, to determine whether the sexually dimorphic production of courtship song is a result of sex-specific properties of these motor neurons, the neuroanatomical and neurochemical properties of the mnDFMs will be compared between the sexes.

## 4.2 Comparing the mnDFMs in Males and Females

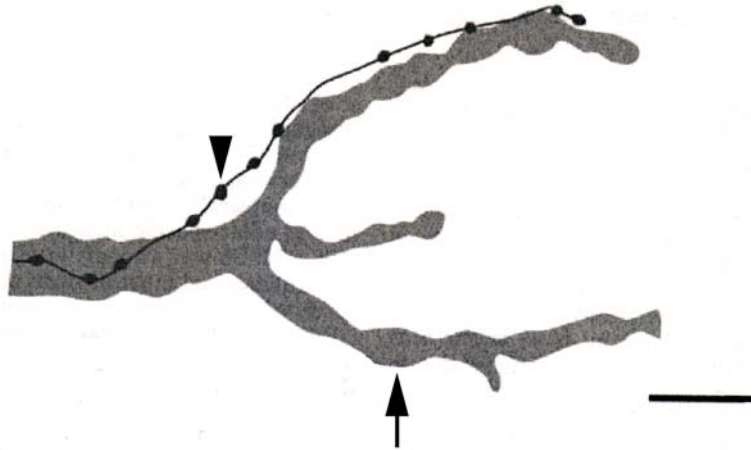
### 4.2.1 Neuroanatomical Characteristics of the mnDFMs

Studies have shown that the sex of a motor neuron can determine its axonal morphology (Lawrence and Johnston, 1986; Gailey *et al.*, 1991; Billeter and Goodwin, 2004). For example, the axonal morphology of a motor neuron which innervates a muscle called the muscle of Lawrence (MOL) in males, and a group of dorsal abdominal muscles in females, is dependent on the sex of the motor neuron (Billeter and Goodwin, 2004). Therefore, the axonal morphology of the mnDFMs in both males and females was examined by incubating the DFM preparations with Cy3-conjugated anti-horseradish peroxidase (HRP). Anti-HRP binds to a *Drosophila* neuron-specific glycoprotein antigen (the glycoproteins are collectively referred to as Nervana) (Jan and Jan, 1982; Wang *et al.*, 1994; Sun and Salvaterra, 1995), and allows the description of neuronal morphology without the use of backfilling dyes, which can be technically challenging. Moreover, using a confocal microscope to visualize the axonal projections of the mnDFMs allows neurons both on the surface and in the plane of the muscle to be detected.

#### mnDFMs

Motor neurons in *Drosophila* adults have one of two different types of bouton morphology: Type I or Type II (Figure 4.3) (Rivlin *et al.*, 2004; reviewed by Gramates and Budnik, 1999). Type I synaptic terminals are large, with a bouton size ranging up to 8  $\mu\text{m}$ , and contain mostly clear synaptic vesicles (Johansen *et al.*, 1989; Koenig and Ikeda, 1989). Type II synapses, on the other hand, are small, with a bouton size of no more than 2  $\mu\text{m}$ , and contain mostly dense core vesicles (Johansen *et al.*, 1989; reviewed by Gramates and Budnik, 1999). All adult muscles examined to date were reported to be innervated by motor

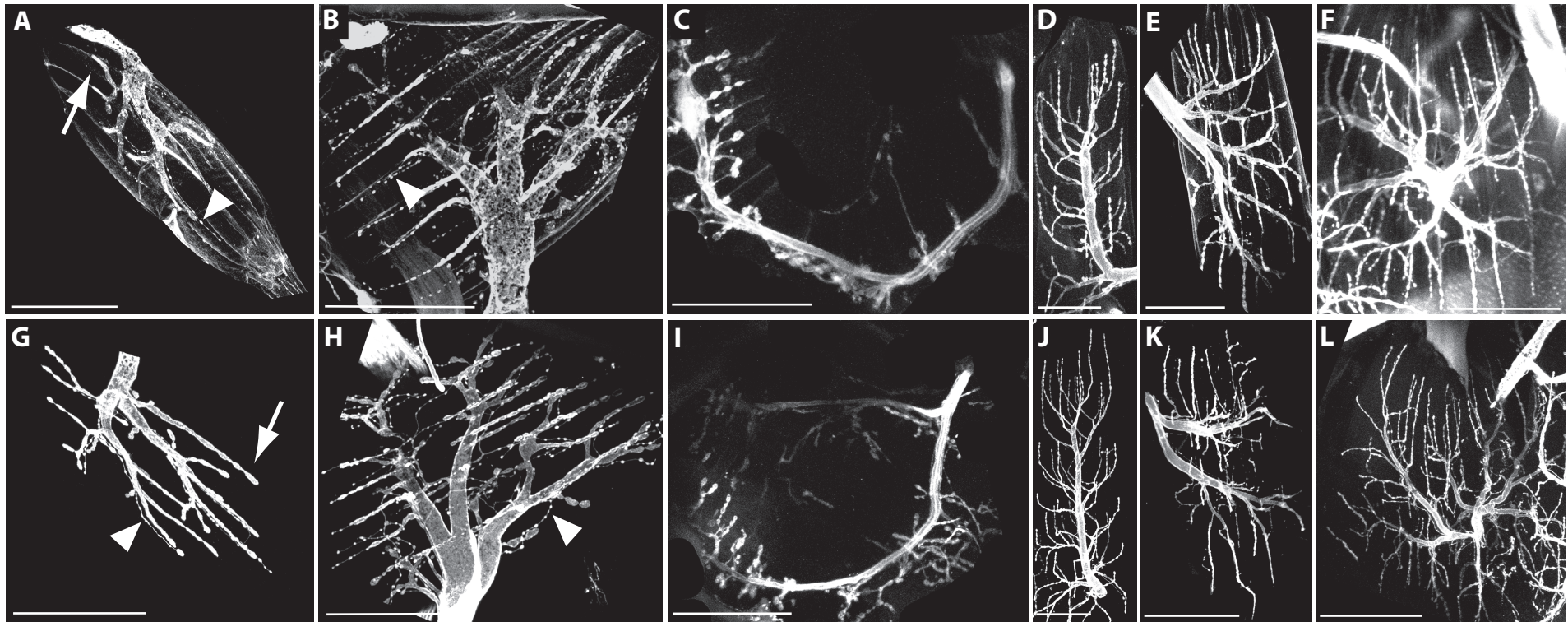
neurons containing both Type I and Type II boutons, respectively (Rivlin *et al.*, 2004); however, the previous study on the mnDFMs described the axonal morphology of the Type I synaptic terminals of only six of the seven mnDFMs, and it is not known whether both males and females were examined (Trimarchi and Schneiderman, 1994).



**Figure 4.3**-Schematic Representation of Synaptic Bouton Types. Schematic representation of the different types of motor neurons synaptic terminals in *Drosophila* (from Rivlin *et al.*, 2004). Type I boutons (arrow) have been reported to be present on all adult muscles, and have a large bouton size, ranging up to 8  $\mu\text{m}$  (Rivlin *et al.*, 2004). Type II boutons (arrowhead) have also been reported to be present on all adult muscles, and have an average bouton size of less than 2  $\mu\text{m}$  (Rivlin *et al.*, 2004).

In this study, a full description of the axonal morphology of both the Type I and Type II motor neurons innervating all seven of DFMs in both males and females was obtained, where mnB1 was visualized for the first time (Figure 4.4 A,G). The axonal morphology of the Type I terminals of the motor neurons innervating B2-B4, AX1a, AX3a and SB were consistent with previous descriptions (Trimarchi and Schneiderman, 1994). All DFMs were innervated by motor neurons with both Type I and Type II synaptic terminals; given that Hoang and Chiba (2001) reported that each motor neuron will predominantly exhibit only one type of synaptic terminal (Type I or Type II), this result suggests that each DFM is innervated by at least two motor neurons (one Type I and one Type II). Indeed, the analysis by King and Tanouye (1983) of the nerve branch innervating B2 found that B2 was innervated by both a large and a small motor axon, which adds still more support to the suggestion that each DFM is innervated by two motor neurons.

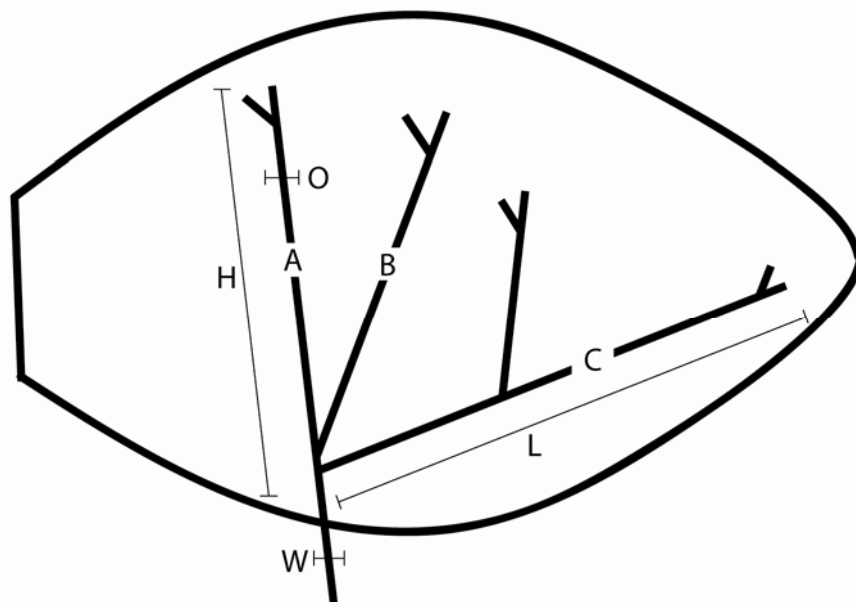




**Figure 4.4**-Morphological comparison of a Subset of the mnDFMs in Males and Females. Maximum Z-projections of confocal images are shown. Anti-HRP-conjugated to Cy3 was used to reveal the neuronal projections (Jan and Jan, 1982). Arrows indicate Type I terminals, arrowheads Type II terminals. Both types of synapse are present on all DFMs. (A-F) Axonal projections of mnDFMs in 5-day-old wild-type CS males: mnB1 (A), mnB2 (B), mnB3/B4 (C), mnAX1a (D), mnAX3a (E), and mnSB (F). (G-L) Axonal projections of mnDFMs in 5-day-old wild-type CS females: mnB1 (G), mnB2 (H), mnB3/B4 (I), mnAX1a (J), mnAX3a (K), and mnSB (L). DFM nomenclature as per Ewing (1979). Scale bar = 50  $\mu$ m.

## Males vs. Females

A qualitative comparison of the branching pattern and axon morphology of the mnDFMs was made between the sexes (Figure 4.4), and no obvious differences were apparent. Thus, in order to determine if more subtle differences between the sexes were present, a quantitative analysis of the Type I terminals of mnB2 was performed. The number of primary and secondary branches, various branch lengths, and Type I bouton size were measured (Figure 4.5, Table 4.1). No significant differences between males and females were observed ( $p > 0.05$ ). Given that no clear differences in axonal morphology were observed between the sexes, these results suggest that the sexually dimorphic production of courtship song is unlikely to be a result of the sex-specific axonal morphology of the mnDFMs.



**Figure 4.5**-Quantitative Measurements of mnB2. Schematic representation of B2 and mnB2. Letters A-C represent the primary branches of mnB2. Other measurements of mnB2 are as follows: the height of primary branch A (H), the length of primary branch B (L), the width of primary branch A (O), and the width of the main axonal branch (W).

Measurement ( <i>n</i> )	Male ( $\mu\text{m}$ )	Female ( $\mu\text{m}$ )
Primary Branches of B2 (8)	$3.0 \pm 0.0$	$3.0 \pm 0$
Secondary Branches of B2 (6)	$5.3 \pm 0.5$	$5.5 \pm 0.5$
Type I Bouton Size (7)	$6.6 \pm 0.8$	$6.8 \pm 0.7$
Measurement W (7)	$22.1 \pm 3.0$	$22.9 \pm 1.9$
Measurement O (7)	$8.6 \pm 0.8$	$8.1 \pm 1.4$
Measurement L (8)	$147.2 \pm 13.0$	$152.2 \pm 9.1$
Measurement H (7)	$77.4 \pm 4.2$	$79.1 \pm 9.1$

**Table 4.1**-Quantitative Comparison of mnB2 in Males and Females. Measurements as described in Figure 4.5 are shown. Data are shown as mean  $\pm$  s.d.

### 4.2.2 Neurochemical Characteristics of the mnDFMs

Although no obvious differences exist between the sexes in the axonal morphology of the mnDFMs, it is possible that neurotransmitter expression at the neuromuscular junction (NMJ) the mnDFMs may be sexually dimorphic. It has been shown that the sex of a sexually dimorphic cluster of neurons in the Abg of the CNS determines the male-specific expression of serotonin in these neurons (Lee and Hall, 2001; Lee *et al.*, 2001; Billeter *et al.*, 2006b). Moreover, the absence of serotonin expression in these neurons may be associated with male-specific infertility (Lee *et al.*, 2001). Thus sex-specific neurotransmitter expression may play a role in sexually dimorphic behaviours.

In this study, the expression of common neurotransmitters at the NMJ of the mnDFMs was investigated by labelling the mnDFMs both with anti-HRP (Jan and Jan, 1982; Wang *et al.*, 1994; Sun and Salvaterra, 1995) and a neurotransmitter-specific antibody to determine its presence or absence.

#### Neurotransmitters at the NMJ

Glutamate is suggested to be the primary excitatory neurotransmitter at the NMJ in *Drosophila* (Jan and Jan, 1976; Johansen *et al.*, 1989). At the larval NMJ, glutamate is present in both Type I and Type II synaptic terminals (Johansen *et al.*, 1989). In the adult NMJ, however, glutamate is only present in Type I synaptic terminals (Rivlin *et al.*, 2004). The only other neurotransmitter found at the adult NMJ to date is octopamine, where it is thought to act primarily as a

neuromodulator (Monastirioti *et al.*, 1995; reviewed by Roeder, 1999). Octopamine is present exclusively in Type II terminals at both the larval and the adult NMJ (Monastirioti *et al.*, 1995; Rivlin *et al.*, 2004).

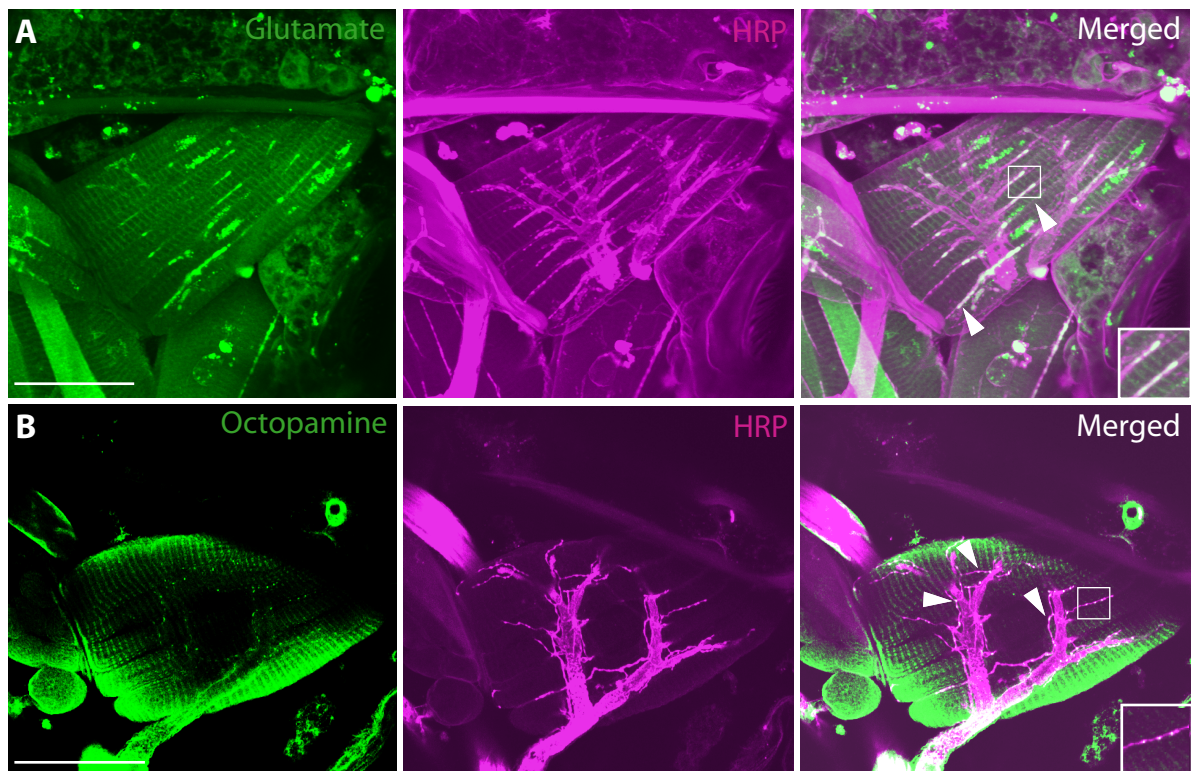
In this study, the expression of the following common neurotransmitters was investigated at the NMJ of the mnDFMs: glutamate, octopamine, serotonin, acetylcholine and dopamine. Glutamate and octopamine were found at the adult NMJ of the mnDFMs, where glutamate was expressed in Type I synaptic terminals only, and octopamine was expressed exclusively in Type II terminals. This finding is in accordance with previous reports of neurotransmitter expression at the NMJ of a set of prothoracic muscles in adults (Table 4.2, Figure 4.6) (Rivlin *et al.*, 2004).

Neurotransmitter	Type I Synaptic Terminal	Type II Synaptic Terminal
Glutamate	+	-
Octopamine	-	+
Serotonin	-	-
Dopamine	-	-
Acetylcholine	-	-

**Table 4.2**-Neurotransmitters present or absent at the NMJ of the mnDFMs. '+' indicates that the neurotransmitter was determined to be present at the NMJ, and '-' indicates that the neurotransmitter was not found at the NMJ.

### Males vs. Females

Of the panel of common neurotransmitters examined in this study, no sexually dimorphic neurotransmitters expression at the NMJ of the mnDFMs was observed. In both males and females, glutamate was found exclusively in Type I synaptic terminals, and octopamine was expressed only by Type II terminals. Therefore, it is unlikely that the sexually dimorphic nature of song production is due to sex-specific neurotransmitter expression at the NMJ of the mnDFMs.



**Figure 4.6-** Neurotransmitter expression at the NMJ of B2. The expression of both glutamate and octopamine in mnB2 was representative of neurotransmitter expression in all direct flight muscles. Anti-HRP-conjugated to Cy3 was used to reveal the neuronal projections (Jan and Jan, 1982). (A) Maximum Z-projection of a confocal image is shown. Anti-glutamate antibody was applied to reveal the expression of glutamate at the NMJ of B2. Glutamate expression was seen only in Type I boutons (arrowheads). White box indicates the region from which the inset image was taken. (B) Z-projection of a confocal image is shown (slice = 2  $\mu\text{m}$ ). Anti-octopamine antibody was used to visualize the expression of octopamine at the NMJ of B2. Octopamine expression was detected only in Type II boutons (arrowheads). White box indicates the region from which the inset image was taken. Scale bar = 50  $\mu\text{m}$ .

### **4.3 Neurobiological Characteristics of the mnDFM in *fruitless* and *doublesex* Mutants**

Mutations in the sex determination genes *fru* and *dsx* have been shown to cause aberrant song phenotypes (Villella and Hall, 1996; Ryner *et al.*, 1996; Villella *et al.*, 1997; Goodwin *et al.*, 2000); however, the neurobiological aetiology of these defects is unknown. Thus, the mnDFMs were examined in *fru* and *dsx* mutant males and compared with the mnDFMs of wild-type males, to investigate the possibility that the song defects of *fru* and *dsx* mutants are a result of aberrant neuroanatomy or neurochemistry of the mnDFMs.

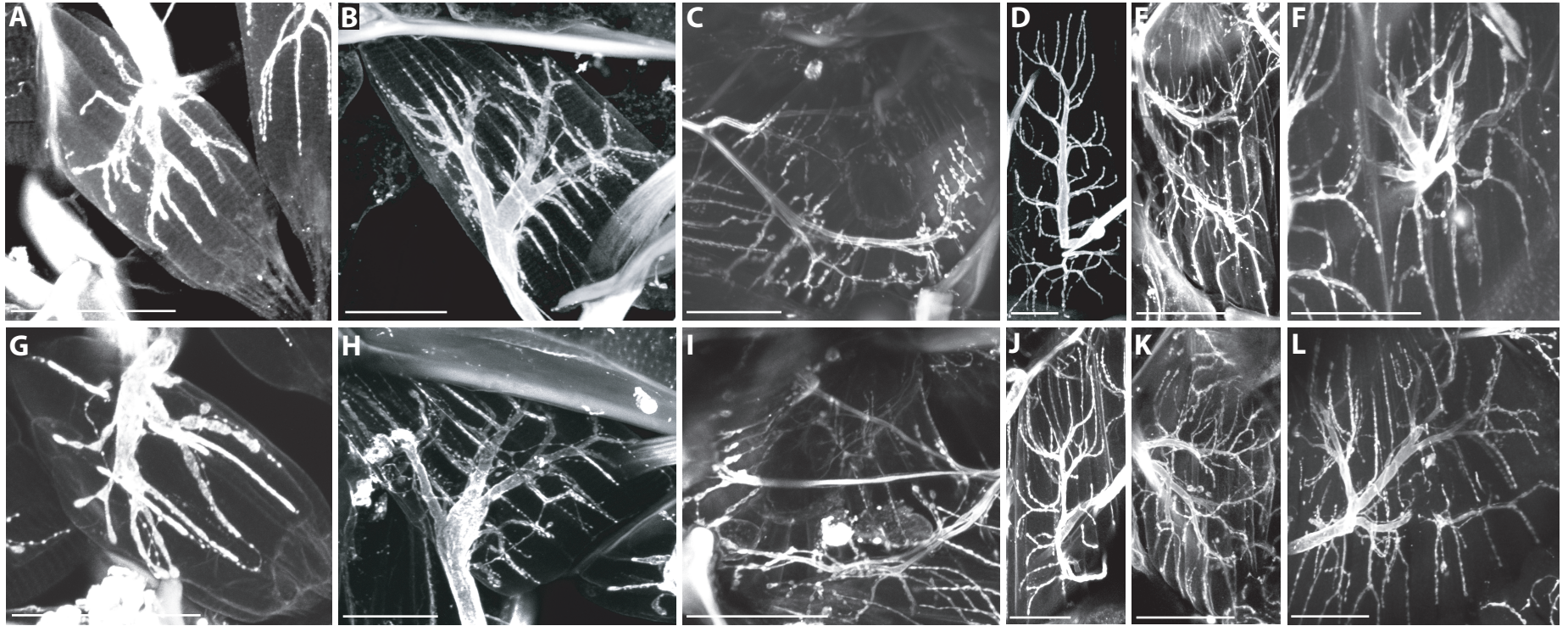
#### **4.3.1 Neuroanatomical Characteristics of the mnDFMs**

mnDFM preparations were incubated with Cy3-conjugated anti-HRP and examined in *fru*<sup>3</sup> homozygote males (*Fru*<sup>M</sup> mutant males) and *In(3R)dsx*<sup>23</sup>/*Df(3R)dsx*<sup>15</sup> heterozygotes (*Dsx* null mutant males) (Figure 4.7). No obvious differences in axonal morphology were apparent in the *fru* or the *dsx* mutants when compared to wild-type males (analysis as in 4.2.1). Therefore, the song defects of *fru* and *dsx* mutant males are not likely a result of aberrant axonal morphology of the mnDFMs.

#### **4.3.2 Neurochemical Characteristics of the mnDFMs**

mnDFM preparations in *fru*<sup>3</sup> homozygous males and in *dsx* null mutant males were incubated both with anti-HRP conjugated to Cy3 and neurotransmitter-specific antibodies, as described in section 4.2.2. In both *fru* and *dsx* mutant males, as in wild-type males, Type I synaptic terminals were found to express glutamate, and Type II terminals to express octopamine. Therefore, the song defects exhibited by *fru* and *dsx* mutants are not likely due to aberrant neurotransmitter expression at the NMJ of the mnDFMs.





**Figure 4.7-** Axonal Morphology of the mnDFMs in *fru* and *dsx* Mutant Males. Maximum Z-projections of confocal images are shown. Anti-HRP conjugated to Cy3 was used to reveal the neuronal projections (Jan and Jan, 1982). (A-F) Axonal projections of mnDFMs in 5-day-old *fru*<sup>3</sup> mutant males: mnB1 (A), mnB2 (B), mnB3/B4 (C), mnAX1a (D), mnAX3a (E), and mnSB (F). (G-L) Axonal projections of mnDFMs in 5-day-old *dsx* null mutant males (genotype *In(3R)dsx*<sup>23</sup>/*Df(3R)dsx*<sup>15</sup>): mnB1 (G), mnB2 (H), mnB3/B4 (I), mnAX1a (J), mnAX3a (K), and mnSB (L). DFM nomenclature as per Ewing (1979). Scale bar = 50  $\mu$ m.

## 4.4 The Importance of Neurochemistry to Song Production

Although the sexually dimorphic production of song does not seem to be linked to the sex-specific expression of a neurotransmitter, the neurotransmitters found at the NMJ of the mnDFMs may still play distinct roles in the production of courtship song.

### Glutamate

Glutamate is the primary excitatory neurotransmitter in *Drosophila*, and is widely expressed throughout the CNS and in all Type I synaptic terminals (Jan and Jan, 1976; Johansen *et al.*, 1989). As a result of this widespread expression, mutants in which glutamate-mediated neurotransmission is disrupted display a number of severe behavioural defects (Hayward *et al.*, 1993; Miklos *et al.*, 1987), and are therefore rendered unsuitable for courtship song analysis in this study.

### Octopamine

Octopamine is an important neuromodulator in many insects, and has been shown to play a role in a number of behaviours, including aggression, egg-laying, locomotion, phototaxis and some aspects of learning and memory (Dudai *et al.*, 1987; O'Dell and Burnet, 1988; O'Dell, 1993; Monastirioti *et al.*, 1996; Baier *et al.*, 2002; Monastirioti, 2003). Octopamine expression in the adult CNS is restricted to approximately 100 neurons, and is present in only Type II synaptic terminals at the NMJ (Monastirioti *et al.*, 1995; Rivlin *et al.*, 2004; Sinakevitch and Strausfeld, 2006). Reduced locomotor activity and female sterility have both been reported in mutants with either a reduction in, or a complete lack of, octopamine (O'Dell *et al.*, 1987; O'Dell and Burnet 1988; Monastirioti *et al.*, 1996); however, these reported behavioural defects are not so severe as to preclude courtship song analysis in octopamine mutants.



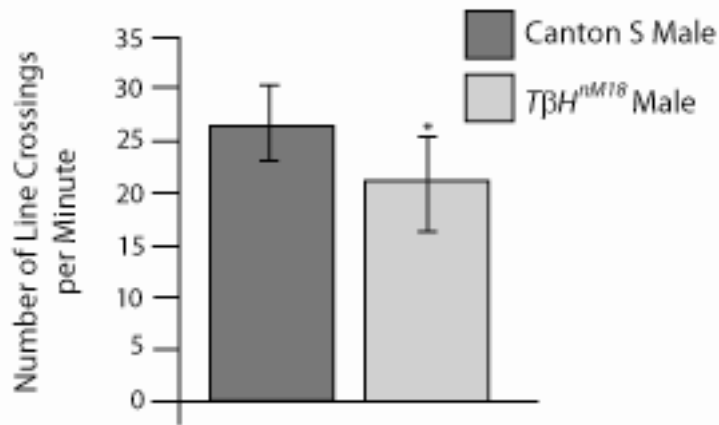
#### **4.4.1 Examination of the Song Production in Octopamine Null Mutants**

Octopamine is synthesized from tyrosine, where tyrosine is first converted by tyrosine decarboxylase into tyramine, and tyramine is then converted into octopamine by Tyramine- $\beta$ -hydroxylase (T $\beta$ H) (Livingstone and Tempel, 1983). Mutants lacking octopamine were created by the imprecise excision of the MF372 *P*-element in the *T $\beta$ H* gene (Monastirioti *et al.*, 1996). This mutation, called *T $\beta$ H*<sup>*nM18*</sup>, results in flies with no detectable octopamine expression, as determined by immunohistochemistry and HPLC (Monastirioti *et al.*, 1996). Homozygous mutant females retain fully developed eggs, however, the mutant flies are viable, and able to walk, fly and mate (Monastirioti *et al.*, 1996). The *T $\beta$ H*<sup>*nM18*</sup> octopamine null mutant strain used in this thesis was verified prior to use by immunohistochemistry.

##### **4.4.1.1 Activity and Courtship Behaviour**

###### **Activity**

It has been suggested that a reduction in, or complete lack of, octopamine causes locomotion defects in both larvae and adult flies (O'Dell and Burnet, 1988; Saraswati *et al.*, 2004). A large reduction in locomotor activity could severely affect the measurement of courtship behaviour and song, therefore, the activity of *T $\beta$ H*<sup>*nM18*</sup> mutant males (in a wild-type CS background) was compared to the activity of wild-type CS males. Activity was measured as described in 2.5.3.1. The activity of the *T $\beta$ H*<sup>*nM18*</sup> males was significantly decreased compared to wild-type CS males ( $n=9$ ,  $p<0.05$ ), where wild-type males had  $27.2 \pm 3.6$  crossings/minute (mean  $\pm$  standard deviation), whereas *T $\beta$ H*<sup>*nM18*</sup> males had  $20.6 \pm 3.8$  crossings/minute (Figure 4.8; Appendix 2); however, the reduction in activity was not as significant as previously reported (O'Dell and Burnet, 1988). Therefore, despite having a reduction in activity compared to wild-type males, the activity of the *T $\beta$ H*<sup>*nM18*</sup> mutant males was sufficiently high to allow an analysis of courtship behaviour and song.



**Figure 4.8**-Activity of CS and  $T\beta H^{M18}$  Males. Activity was measured as the number of line crossings per minute for wild-type CS males, and octopamine null mutant  $T\beta H^{M18}$  males. \* indicates a significant difference from wild-type CS males (n=9,  $p<0.05$ ).

### Courtship Behaviour

$T\beta H^{M18}$  mutant males are not sterile, though it has been suggested that a reduction in octopamine is linked to poor mating success (O'Dell, 1993). As shown in the previous section, the  $T\beta H^{M18}$  mutant males have a significant reduction in activity; therefore, the courtship behaviour of these mutant males was measured, to ensure sufficient amounts of wing extension and song production were attempted to permit subsequent analyses.

Despite the fact that the activity of the  $T\beta H^{M18}$  males was only 78% of that seen in wild-type males, the CI was not significantly different (n=13;  $p>0.05$ ). Table 4.3 shows the CI of both wild-type males and  $T\beta H^{M18}$  males; the CI of wild-type CS males was  $88.8 \pm 3.1$  (mean  $\pm$  standard error of the mean), and the CI of  $T\beta H^{M18}$  males was  $80.9 \pm 3.5$  (Appendix 3). Other behavioural parameters pertaining to song production were measured in addition to CI, including the WEI and SI (Table 4.3). The WEI of the  $T\beta H^{M18}$  mutant males was significantly decreased when compared to the WEI of wild-type CS males ( $p<0.05$ ; n=13); however, the SI was not significantly different. Therefore, although the  $T\beta H^{M18}$  males do not perform as much wing extension as wild-type males, once the wing was extended, no obvious defects in the initiation of song production were observed. Thus, an analysis of various parameters of courtship song was performed.

Genotype	<i>n</i>	CI	WEI	SI	SBPM	PTPM	MPPT	IPI (ms)
Canton S (XY)	6	88.8 ± 3.1	55.6 ± 4.6	93.2 ± 3.4	24.1 ± 3.5	20.1 ± 2.1	8.0 ± 0.36	31.5 ± 3.0
<i>TβH<sup>nM18</sup></i> (XY)	13	80.9 ± 3.5	32.1 ± 2.1*	78.2 ± 7.0	14.6 ± 1.3*	13.8 ± 1.3*	8.2 ± 0.16	34.0 ± 1.8*

**Table 4.3**-Courtship behaviour and song analysis of *TβH<sup>nM18</sup>* octopamine mutant males. CI, WEI, SI, SBPM, and PTPM are shown as mean ± s.e.m. MPPT and IPI are shown as the mean of *n* intramale means ± s.e.m. \* Indicates a significant difference (*p*<0.05). Behavioural recordings performed at 25°C.

### Song Analysis

Courtship song parameters were measured in wild-type CS males and *TβH<sup>nM18</sup>* octopamine mutant males, as shown in Table 4.3 (Appendix 3). SBPM, PTPM, MPPT and the IPI are shown. A significant decrease in SBPM and PTPM was observed in *TβH<sup>nM18</sup>* mutant males when compared to wild-type CS males (*p*<0.05). This difference suggests that the *TβH<sup>nM18</sup>* males produced less sine song and pulse song bouts per minute than wild-type CS males; however, it seems likely that the decreased number of SBPM and PTPM reflects the decreased amount of wing extension (and decreased amount of total song) observed in these males. Indeed, the decrease in SBPM and PTPM seems to be equivalent to the percentage difference in WEI between wild-type CS and *TβH<sup>nM18</sup>* males. More support for the suggestion that the decreased SBPM and PTPM were a result of decreased wing extension comes from the finding that the MPPT were not significantly lower in *TβH<sup>nM18</sup>* mutant males. Thus although there were fewer PTPM in *TβH<sup>nM18</sup>* mutant males, these males were capable of producing pulse trains that were wild-type in length. The final song parameter recorded was the IPI, which was significantly increased in *TβH<sup>nM18</sup>* mutant males (*n*=13; *p*<0.05; at 25°C). This difference was not due to an increased number of cycles per pulse (CPP) during pulse song, as no significant differences in CPP were found between wild-type males and *TβH<sup>nM18</sup>* males (*n*=13; *p*>0.05). This suggests a role for octopamine in the modulation of the IPI during courtship song production in males.

## 4.5 Discussion

This chapter investigated the neuromuscular basis for the sex-specific production of courtship song by describing the axonal morphology of the mnDFMs and the neurotransmitter expression at the NMJ of the mnDFMs in both males and females. Moreover, the importance of octopamine to courtship song production was investigated. These findings and their implications are discussed below.

### 4.5.1 *mnDFMs*

#### Axonal Morphology

In this study, the use of Cy3-conjugated anti-HRP to reveal axonal projections, combined with the resolving power of the confocal microscope, permitted a detailed analysis of the mnDFMs not possible using previous methods. The motor neuron innervating B1 was visualized for the first time; moreover, all DFMs were found to be innervated by a minimum of two motor neurons, one glutamatergic, and one octopaminergic. Although the comparison of the axonal morphology of the mnDFMs between males and females yielded no clear differences between the sexes, these findings provide an improved description of the mnDFMs, which will ultimately provide the foundation for future studies of the neuromuscular basis for courtship song production. In addition, the findings in this chapter have demonstrated that gross neuroanatomical and neurochemical differences between the sexes are not responsible for the sex-specific production of courtship song, suggesting that the relevant differences exist elsewhere in the CNS.

It is important to note, however, that subtle differences in neurochemistry or neuronal function, beyond the level of examination in this study, may yet exist in the mnDFMs of males and females. For example, larval locomotion is sexually dimorphic (Glossop and Shepherd, 1998), where Lnenicka *et al.*, 2006 propose that this dimorphism is a consequence of increased neurotransmitter release by female synaptic boutons. This increased neurotransmitter release was not coupled to any differences in neurotransmitter type or bouton morphology and/or size, but is physiologically significant, and potentially leads to a sexually dimorphic behaviour. In the future, electrophysiological recordings could be

performed on the mnDFMs of males and females, where comparing the activity of the mnDFMs would reveal any such differences in the mnDFMs which potentially contribute to the sexually dimorphic production of song.

Another subtle difference between the mnDFMs of males and females not examined in this study could be the dendritic projections of the mnDFMs within the CNS. During metamorphosis, the dendritic projections of motor neurons and other neurons are dramatically re-modelled according to a variety of signals, both intra- and extracellular (reviewed by Consoulas *et al.*, 2002; Williams and Truman, 2004). It is therefore possible that the dendritic fields of the mnDFMs within the CNS are re-modelled in a sexually dimorphic manner during metamorphosis, to adjust for sex-specific synaptic inputs, ultimately leading to differences in sex-specific behaviours in males and females. To determine whether this is in fact the case, the mnDFMs could be backfilled by placing HRP crystals on the DFMs (as performed by Trimarchi and Schneiderman, 1994), and the projections of the motor neurons within the CNS could be visualized in both males and females.

### Neurotransmitter Expression

Neurotransmitter expression at the NMJ of the mnDFMs was examined in both males and females, where glutamate, an excitatory neurotransmitter, was present exclusively in Type I synaptic terminals, and octopamine, an important neuromodulator, was found only in Type II terminals. Although no sex-specific neurotransmitter expression was found, these findings contribute to the current knowledge of the neurochemistry of courtship song production. As discussed for the axonal morphology of the mnDFMs, subtle differences between males and females may exist that were not detected in this study. For example, only the expression of common neurotransmitters was investigated; however, other neuropeptides, such as proctolin, have been shown to have a very limited presence at the larval NMJ (Anderson *et al.*, 1988). Thus neuropeptide expression at the NMJ may be sexually dimorphic, but any dimorphism in an uncommon neurotransmitter or neuromodulator would not have been detected in this study. In addition, the expression of glutamate and octopamine was not quantified, and it is therefore possible that the amount of transmitter present and/or released at the NMJ of males and females was sexually dimorphic; and as

discussed above, these differences may lead to sexually dimorphic behaviours (Lnenicka *et al.*, 2006).

### **4.5.2 Octopamine**

#### **General Behaviour**

This part of the chapter examined the role of octopamine in the modulation of courtship song. As previous studies have demonstrated a role for octopamine in a number of behaviours, including activity and mating success (O'Dell and Burnet, 1988; O'Dell, 1993), the activity and courtship behaviour of the octopamine mutants were examined to determine their suitability for courtship song analysis. Although the octopamine mutant males showed a significant decrease in activity, this difference did not prevent them from performing wild-type amounts of courtship behaviour. Thus octopamine does not significantly affect the performance of male courtship behaviour. Previous studies suggesting that decreased locomotion and poor mating success were a result of decreased octopamine expression were performed in *inactive (iav)* mutants, which have a reduced amount of both tyramine and octopamine (O'Dell *et al.*, 1987; O'Dell and Burnet, 1988; O'Dell, 1993). More recent work, however, has suggested that the decreased activity of *iav* mutants is primarily a result of decreased tyramine levels, not decreased levels of octopamine (McClung and Hirsh, 1999). Indeed, this may also be true for courtship behaviour, as this work in this thesis has shown that the amount of courtship behaviour performed by octopamine null mutant males was not significantly decreased.

#### **Courtship Song**

Given that octopamine does not affect the amount of courtship behaviour performed by the male, behaviours directly related to courtship song production were examined in more detail, and courtship song was analyzed. Wing extension was reduced in octopamine mutant males, however, the time spent singing once the wing was extended was not significantly decreased. This demonstrates a role for octopamine in the neural control of wing extension, but not necessarily in the initiation of song production. In locusts, the injection of octopamine into the metathoracic ganglion causes the initiation of the complete flight-motor pattern (Sombati and Hoyle, 1984). Similar results have been reported in the moth,

*Manduca sexta*, where the injection of octopamine into the mesothoracic ganglion was sufficient to trigger the flight motor output (Claassen and Kammer, 1986). Taken together, these findings suggest that octopamine could play a similar role in *Drosophila* in the initiation of flight motor patterns, and so may explain the decreased wing extension of the octopamine mutant males.

Other song parameters measured included SBPM, PTPM, MPPT, and IPI. With the exception of IPI, all song parameters were essentially normal in  $T\beta H^{nM18}$  mutant males, suggesting that octopamine is not a critical regulator of many aspects of courtship song. In the case of IPI, however, the lack of octopamine affected the timing of the pulses. Octopamine is known to modulate the central pattern generators for a number of rhythmic behaviours (Sombati and Hoyle, 1984; Ramirez and Pearson, 1991; Mizutani *et al.*, 2002; Fox *et al.*, 2006). As discussed above, the injection of octopamine into specific regions of the CNS is sufficient to elicit the flight motor of both the locust and the moth; in addition, the rhythmic respiratory movements of the locust have also shown to be altered upon the application of octopamine (Sombati and Hoyle, 1984). Still more evidence of the modulation of central pattern generation by octopamine has been found in *Drosophila* larvae, where octopamine null larvae have more episodes of non-rhythmic neuronal activity than do wild-type larvae, which may indicate an altered motor output by central neurons (Fox *et al.*, 2006). Thus the aberrant pulse-timing of octopamine null mutant males adds further support to the hypothesis that octopamine is a critical modulator of central pattern generators, including that required for courtship song production.

To further confirm the requirement for octopamine in wing extension, and in modulating the length of the IPI, a promoter-GAL4 fusion of the *tyrosine decarboxylase 2* (*Tdc-2*) gene (which converts tyrosine into tyramine) can be used (Livingstone and Tempel, 1983; Cole *et al.*, 2005). *Tdc-2*-GAL4 appears to drive the expression of GAL4 in most octopamine neurons (Cole *et al.*, 2005); therefore, the wing extension and IPI defects of the  $T\beta H^{nM18}$  octopamine null mutant could be rescued by driving the expression of UAS-*T\beta H* with *Tdc-2*-GAL4 (Monastirioti, 2003; Cole *et al.*, 2005). Moreover, the contribution of the octopamine- and tyramine-expressing neurons to wing extension and IPI length could also be assayed by conditionally disrupting the function of octopamine neurons by crossing *Tdc-2*-GAL4 to UAS-*shi<sup>ts</sup>* (Kitamoto, 2001; Cole *et al.*, 2005).

Finally, it would be interesting to determine whether the *Tdc-2*-expressing neurons are sufficient to induce the flight motor pattern or courtship song motor pattern in *Drosophila* using *Tdc-2*-GAL4 to drive the expression of an optically gated ion channel (Lima and Miesenböck, 2005). If the light-induced activation of the *Tdc-2*-expressing neurons, as described by Lima and Miesenböck (2005), initiates either the flight- or the courtship song-motor pattern, it would suggest that octopamine in *Drosophila melanogaster*, as in other invertebrates, is involved in the control of wing extension and the modulation of the central pattern generator for flight and/or song (Sombati and Hoyle, 1984; Claassen and Kammer, 1986).

### 4.5.3 Conclusions

The aim of this chapter was to investigate the possibility that the song defects of *fru*<sup>M</sup> and *fru*<sup>Atra</sup> females were due to the aberrant neuroanatomical or neurochemical properties of the motor neurons responsible for beating the wing during courtship song. Moreover, since *fru* and *dsx* were both found to be required for the specification of courtship song, these motor neurons were also investigated in *fru* and *dsx* mutants, to identify any *fru*- and/or *dsx*-regulated structures contributing to the sex-specific production of courtship song. In fact, no significant dimorphisms between the sexes were found, nor between wild-type males and *fru*- and *dsx*-mutant males, suggesting that the neurobiological aetiology of the song defects in these females (and *fru* and *dsx* mutant males) must lie elsewhere in the CNS. Given that studies using male-female mosaics have shown that the ventral thoracic ganglia of the CNS are critical to the production of courtship song (von Schilcher and Hall, 1979), a detailed examination of this region will be performed in the next chapter, to identify any dimorphisms which may contribute to the sex-specific production of courtship song.



## **5 Co-Expression of Fru<sup>M</sup> and Dsx, and the Mesothoracic Ganglion**

## 5.1 Introduction

In the previous chapter, a detailed examination of the NMJ of the DFMs was performed, and no obvious differences between the sexes were observed, suggesting that the neurobiological basis for the sexually dimorphic production of courtship song is not due to sexually dimorphic motor output resulting from the sex-specific innervation of the DFMs. Thus the critical differences between the sexes may instead be due to sex-specific neural circuits governing courtship song production.

Sex mosaic studies have shown that in gynandromorphs with a male head, the ventral thoracic ganglia of the CNS must also be male for normal song production to occur (von Schilcher and Hall 1979). These results suggest that the neural foci for courtship song lie in the thoracic ganglia of the CNS, and also that the sex of this region is key to song production. Intriguingly, sex determination genes *fru* and *dsx* are both expressed in the ventral thoracic ganglia of the CNS. Moreover, a critical requirement for both *fru* and *dsx* in the specification of the courtship song was demonstrated in Chapter 3, suggesting that these genes act in concert, or in parallel, to specify properties of the CNS underlying the sex-specific production of courtship song. Thus in this chapter the thoracic ganglia of the CNS will be examined in detail, to document any sexually dimorphic properties of this region, and to investigate the relative contributions of *fru* and *dsx* to the creation of these differences.

## 5.2 Co-Expression of Fru<sup>M</sup> and Dsx in the CNS

Although it has been suggested in the past that *dsx* is responsible for the development of the primary sexual characteristics of the fly, and that *fru* directs the sex-specific development of the CNS, mounting evidence suggests that the developmental roles of *fru* and *dsx* are not so easily separated (reviewed by Billeter *et al.*, 2006a). First, Taylor and Truman (1992) showed that *dsx* was responsible for the creation of a male-specific population of neurons in the Abg of the CNS. Second, *dsx* is expressed in discrete locations in the CNS (Lee *et al.*, 2002). Thus the role of *dsx* in sexually dimorphic development is not limited to tissues outside of the CNS; rather, it makes an important contribution to specification of sex in the CNS.

Both *fru* and *dsx* are expressed in the CNS, and mutations in either gene are associated with defects in male courtship behaviour and song (McRobert and Tompkins, 1985; Taylor *et al.*, 1994; Villella and Hall, 1996; Ito *et al.*, 1996; Ryner *et al.*, 1996; Villella *et al.*, 1997; Lee *et al.*, 2000; Goodwin *et al.*, 2000; Lee *et al.*, 2001; Lee and Hall, 2001; Lee *et al.*, 2002; Demir and Dickson, 2005); indeed, results in Chapter 3 showed that both Fru<sup>M</sup> and Dsx<sup>M</sup> are required for the specification of courtship song. However, apart from a recent study by Billeter *et al.* (2006b), no studies have investigated whether *fru* and *dsx* act in parallel (if in different neurons), or in concert (if in the same neurons) to specify a neural substrate for male courtship behaviours and courtship song.

Dsx expression in the CNS is first detected in third instar larvae, with the maximum expression in 1- to 2-day-old pupae (Lee *et al.*, 2002). The number of cells in which Dsx is expressed remains constant throughout later pupal stages and into adulthood, but the expression levels are much lower than in 1- to 2-day-old pupae (Lee *et al.*, 2002). Similarly, Fru<sup>M</sup> proteins are first detected in the CNS of late third instar wandering larvae, and reach the maximum levels of expression in 2-day-old pupae (Lee *et al.*, 2000). The number of neurons in which Fru<sup>M</sup> is expressed is higher in adults than in pupae, however, the levels of expression are decreased (Lee *et al.*, 2000). Based on the temporal expression profiles of each protein, then, the CNSs of male 2-day-old pupae and 5-day-old adults were examined to determine whether Fru<sup>M</sup> and Dsx were co-expressed.

### 5.2.1 Posterior Brain

Sex mosaic studies have shown that the neural foci for male behaviours in general reside within the dorsal posterior region of the CNS (Figure 5.1) (Hall, 1977; Hall, 1979; Ferveur and Greenspan, 1998). Specific behaviours associated with this region include tapping, licking and wing extension (Hall, 1977; Hall, 1979; Ferveur and Greenspan, 1998). Figure 5.2 shows the co-expression of Fru<sup>M</sup> and Dsx in the posterior brain of a 2-day-old wild-type male pupa. Co-localization was found in a subset of neurons of the pC1 and pC2 clusters of Dsx-expressing cells in the posterior brain (nomenclature as per Lee *et al.*, 2002) from the pupal stage onward. The number of neurons co-expressing Fru<sup>M</sup> and Dsx in 2-day-old pupae was  $34 \pm 4.6$  (mean  $\pm$  s.d.) in the pC1 cluster, and  $22.8 \pm 5.6$  (mean  $\pm$  s.d.) in the pC2 cluster (n=5; pupal counts as per J.C. Billeter).

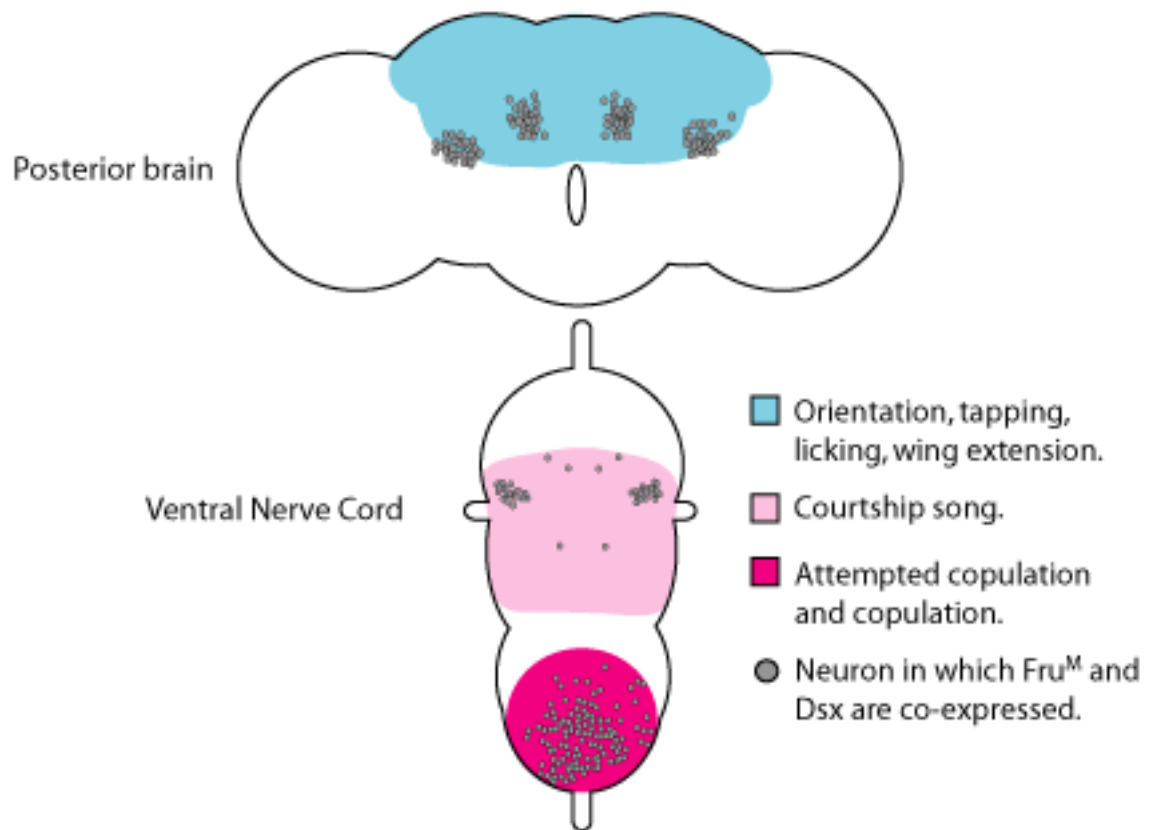
The co-expression of Fru<sup>M</sup> and Dsx in this region implies that the two may act in concert to specify a neural substrate required for the initiation of male courtship, in addition to tapping, licking and wing extension. Mutations in either *dsx* or *fru* cause decreased levels of courtship towards females, as well as decreased tapping, licking and wing extension (Taylor *et al.*, 1994; Ryner *et al.*, 1996; Villella and Hall, 1996; Villella *et al.*, 1997). Moreover, results in Chapter 3 showed that the initiation of courtship behaviour in females expressing Fru<sup>M</sup> was highly aberrant, and that many of these females were not able to progress from early courtship steps to tapping and licking. Together, these results support the suggestion that Fru<sup>M</sup> and Dsx act in concert to specify a number of male-specific behaviours.

### 5.2.2 Abdominal Ganglion

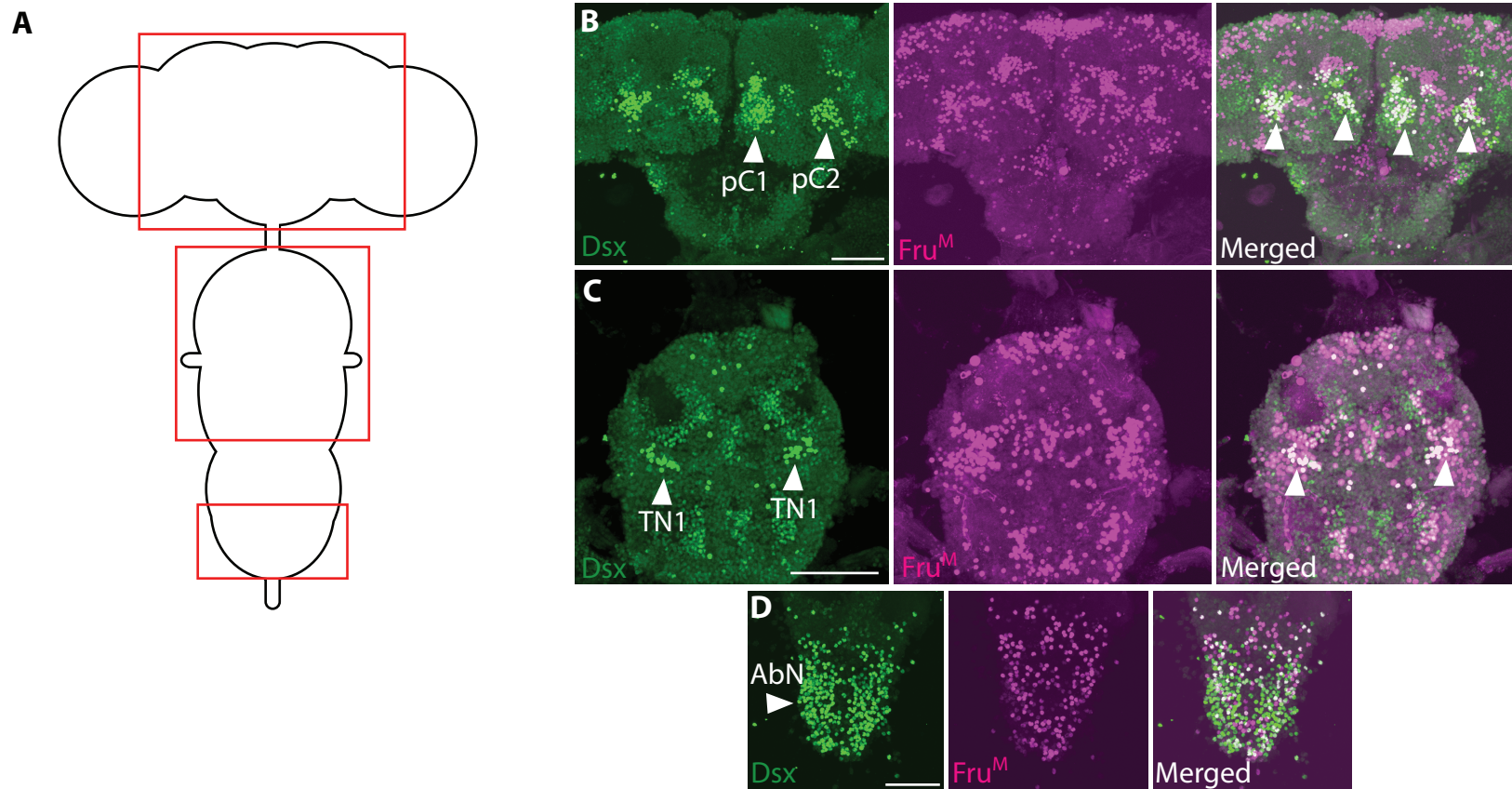
A number of studies have shown that the sex of the Abg is critical to the ability of the male both to attempt copulation and to copulate (Figure 5.1) (Hall, 1977; Hall, 1979; Ferveur and Greenspan, 1998). Moreover, several reports have identified *dsx*- and/or *fru*-regulated sexually dimorphic characteristics of this region (Taylor and Truman, 1992; Lee and Hall, 2000; Lee *et al.*, 2001; Billeter *et al.*, 2006b). Figure 5.2 shows the co-expression of Fru<sup>M</sup> and Dsx in the Abg of a 2-day-old wild-type male pupa (this co-expression is reported in Billeter *et al.*, 2006b). Co-localization occurred in a subset of *dsx*-expressing neurons called the AbN cluster (nomenclature as per Lee *et al.*, 2002), from the pupal stage onward. The number of neurons co-expressing Fru<sup>M</sup> and Dsx was  $112 \pm 16$  neurons ( $n=5$ ; mean  $\pm$  s.d.; pupal counts as per J.C. Billeter), and was not significantly different in 5-day-old adults ( $n=5$ ;  $p>0.05$ ).

Again, the co-localization of Fru<sup>M</sup> and Dsx in the Abg suggest that the two may act together in the Abg to generate a neural substrate for attempted copulation and copulation. Indeed, Billeter *et al.* (2006b) showed that both Fru<sup>M</sup> and Dsx are required for the specification of a male-specific population of serotonergic neurons. The loss of this male-specific population of serotonergic neurons is correlated with fertility defects in *fru* mutant males, although it has also been suggested that this defect could be due to the loss of these neurons in *fru* mutants (Lee *et al.*, 2001; Lee and Hall, 2001; Billeter *et al.*, 2006b). Thus, Fru<sup>M</sup>

and *Dsx* appear to act together in the Abg to direct the creation of a sexually dimorphic population of neurons that is implicated in male fertility.



**Figure 5.1**-*Fru<sup>M</sup>* and *Dsx* are Co-Expressed in Regions of the CNS Implicated in Sex-Specific Behaviours. Schematic representation of the CNS (posterior of brain, ventral view of VNC; anterior at the top). Regions of the CNS implicated by male-female mosaic studies in the performance of male-specific behaviours are highlighted: blue indicates the region involved in orientation, tapping, licking and wing extension; pink indicates the region thought to be involved in song production; dark pink indicates the region implicated in attempting copulation and copulation. Neurons in which *Fru<sup>M</sup>* and *Dsx* are co-expressed are present in each of these regions, as shown by gray circles. Adapted from Billeter *et al.* (2006a).



**Figure 5.2-** Co-Expression of  $Fru^M$  and  $Dsx$  in the CNS. (A) Schematic representation of the CNS (from Billeter *et al.*, 2006a). Red boxes (top to bottom) outline the corresponding regions in the CNS from which the accompanying confocal images were taken. (B-D) Maximum Z-projections of confocal images are shown. Whole mount confocal images from 2-day-old pupal wild-type males co-labelled with anti- $Fru^M$  and anti- $Dsx$ . Co-localization was seen in four distinct regions of the CNS: a subset of neurons in the pC1 and pC2 clusters of  $dsx$ -expressing neurons in the posterior brain, where  $34 \pm 4.6$  neurons (mean  $\pm$  s.d.) per hemisegment in pC1, and  $22.8 \pm 5.6$  neurons per hemisegment in pC2 showed co-localization (B), in a subset of neurons in the TN1 cluster of  $dsx$ -expressing neurons in the Msg (C), and in a subset of the AbN cluster of  $dsx$ -expressing neurons in the Abg (D) (all  $dsx$  nomenclature as per Lee *et al.*, 2002). (B) Dorsal view with anterior at the top. (C,D) Ventral view, anterior at the top. Scale bar = 50  $\mu$ m.

### 5.2.3 Mesothoracic Ganglion

In gynandromorphs with a male head, the ventral thoracic ganglia of the CNS (including the *Msg*) must also be male for wild-type courtship song (Figure 5.1) (von Schilcher and Hall, 1979). This data suggests that the neural foci of courtship song are located in the ventral thoracic ganglia, and that the sex of this region is critical to song production. Figure 5.2 shows the co-expression of *Fru<sup>M</sup>* and *Dsx* in the ventral thoracic ganglia of the CNS of a 2-day-old wild-type male pupa, specifically the *Msg*. Co-localization occurred in a subset of *Dsx*-expressing neurons located in the *Msg* called the TN1 cluster (nomenclature as per Lee *et al.*, 2002), and was found from the pupal stage onward. The number of neurons co-expressing *Fru<sup>M</sup>* and *Dsx* in 2-day-old male pupae was  $17.4 \pm 1.7$  per hemisegment (mean  $\pm$  s.d.), and was not significantly different in 5-day-old adults ( $n=10$ ;  $p>0.05$ ).

The co-localization of *Fru<sup>M</sup>* and *Dsx* in the *Msg* is provocative, given the importance of this region to song production (von Schilcher and Hall, 1979). In addition, both *Fru<sup>M</sup>* and *Dsx<sup>M</sup>* are required for song production, suggesting that they act in concert in the *Msg* to specify courtship song production. Therefore, a detailed examination of the *Msg* will be performed, to determine whether any dimorphisms exist, and if so, what roles *fru* and *dsx* play in their creation.

## 5.3 The Mesothoracic Ganglion

The co-expression of *Fru<sup>M</sup>* and *Dsx* in the *Abg* was described in a recent paper, where both *fru* and *dsx* were shown to be required for the specification of a male-specific cluster of serotonergic neurons in this region (Billeter *et al.*, 2006b). Given the critical importance of the sex of the ventral thoracic ganglia (including the *Msg*) to song production and the requirement for both *fru* and *dsx* in courtship song production, the co-localization of *Fru<sup>M</sup>* and *Dsx* in this region suggests that as with the *Abg*, sexually dimorphic developmental mechanisms may also operate in the *Msg*, ultimately contributing to the sex-specific nature of courtship song production.

Previous studies have demonstrated that sexually dimorphic neuronal populations exist in specific regions of the brain. In the *Abg*, males have more

neurons in the Abg than females as a result of sex-specific neurogenesis (Truman and Bate, 1988; Taylor and Truman, 1992). Likewise, Kimura *et al.* (2005) reported a greater number of neurons in the mAL cluster of Fru<sup>M</sup>-expressing neurons in the brain of males. Therefore, the number of neurons in the Msg was quantified in both males and females, to determine whether a sexually dimorphic population of neurons is present in this region.

### 5.3.1 Is the Mesothoracic Ganglion Sexually Dimorphic?

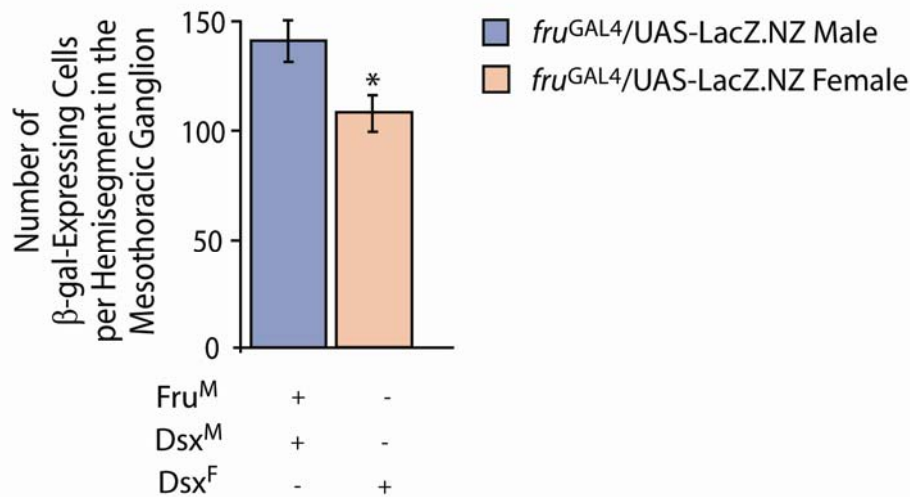
*fru*<sup>GAL4</sup> is a driver with expression in all Fru<sup>M</sup> neurons as a result of the insertion of yeast transcriptional activator GAL4 into the *fru* locus (Stockinger *et al.*, 2005; Figure 3.1); in addition, *fru*<sup>GAL4</sup> drives significant expression in the Msg. Although Fru<sup>M</sup> proteins are expressed exclusively in males, *fru*<sup>GAL4</sup> directs the expression of a reporter in both males and females, and allows the number of Fru<sup>M</sup> neurons in a given region to be quantified in both sexes.

*fru*<sup>GAL4</sup> was used to drive the expression of GAL4-responsive *UAS-LacZ.NZ*, and the number of  $\beta$ -Gal positive neurons were counted in 5-day-old adult males and females. Figure 5.3 shows the number of  $\beta$ -Gal positive neurons in the Msg of both sexes. Males had significantly more neurons in the Msg, with  $136.4 \pm 3.3$  neurons per hemisegment (mean  $\pm$  s.d.), compared to  $111.6 \pm 3.1$  neurons per hemisegment in females ( $n=10$ ;  $p<0.05$ ). A previous study using this driver did not find any significant difference in neuron number between the sexes (Stockinger *et al.*, 2005); however, the dimorphism was most likely missed as a result of the small number of specimens counted, and the age of the flies at dissection.

Previously, Lee *et al.* (2000) reported that the number of neurons expressing *fru*'s P1 transcripts in the Msg was sexually dimorphic. Intriguingly, similar sex-specific neuronal populations expressing *fru*'s P1 transcripts were also found in the posterior brain and the Abg, suggesting that all three regions in which Fru<sup>M</sup> and Dsx are co-expressed contain sexually dimorphic populations (Lee *et al.*, 2000). Further support for the hypothesis that a dimorphic number of neurons is present in the Msg is the fact that a sex-specific population of *dsx*-expressing neurons has been identified in the Msg (Lee *et al.*, 2002). Together with the previous findings then, the results in this chapter suggest that a sexually



dimorphic population of neurons does in fact exist in the *Msg*. Next, the relative roles of *fru* and *dsx* in the creation of this sexually dimorphic population of neurons were investigated.



**Figure 5.3**-Sexually Dimorphic Neuron Numbers in the *Msg*. Mean number of nuclei expressing  $\beta$ -Gal per hemisegment ( $\pm$  s.d.) in the *Msg* of the CNS in 5- to 7-day-old adult males and females. Mean number of nuclei calculated from 10 hemisegments. *fru*<sup>GAL4</sup> drives expression of  $\beta$ -Gal in both males and females. \* denotes a significant difference ( $p < 0.05$ ). Presence or absence of  $Fru^M$ ,  $Dsx^M$  and  $Dsx^F$  are noted below each histogram bar.

### 5.3.2 The Roles of *dsx* and *fru* in Creating a Sexually Dimorphic Population of Neurons in the Mesothoracic Ganglion

$Fru^M$  and *Dsx* are each responsible for the creation of sexually dimorphic populations of neurons elsewhere in the CNS (Taylor and Truman, 1992; Kimura *et al.*, 2005). Taylor and Truman (1992) demonstrated a critical role for  $Dsx^M$  in prolonging neuroblast divisions in the *Abg*, resulting in more neurons in the male *Abg*. Similarly, Kimura *et al.*, (2005) showed that the expression of  $Fru^M$  in a cluster of neurons in the brain could protect against programmed cell death, again resulting in more neurons in males. Thus the difference in *fru*<sup>GAL4</sup>-positive neurons between males and females in the *Msg* could arise as a result of the individual contributions of either  $Fru^M$  or *Dsx*, or as a result of the combined actions of both  $Fru^M$  and *Dsx*. Therefore, the contributions of *fru* and *dsx* to the creation of a sexually dimorphic population of *fru* neurons in the *Msg* were determined.

### 5.3.2.1 Is Fru<sup>M</sup> Sufficient to Create a Sexually Dimorphic Mesothoracic Ganglion?

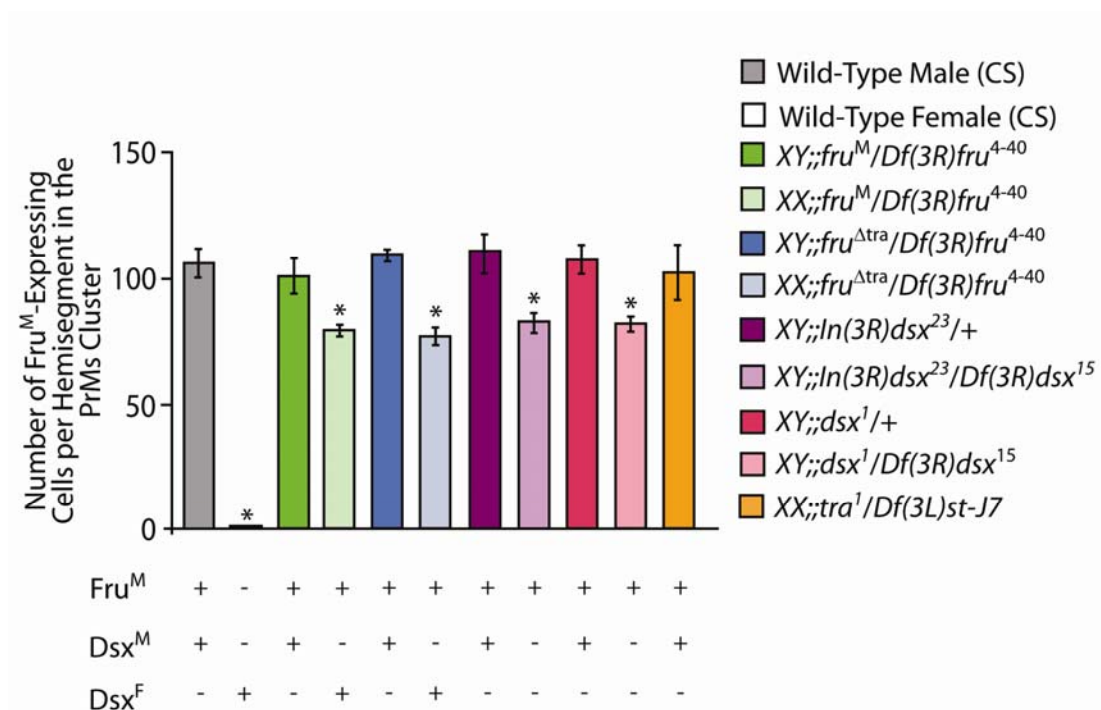
In a specific region of the CNS, Fru<sup>M</sup> expression alone can protect neurons from programmed cell death, creating a sexually dimorphic population of neurons in the brain (Kimura *et al.*, 2005). To determine whether the expression of Fru<sup>M</sup> alone is responsible for the creation of the male-specific population of *fru* neurons in the Msg, the number of Fru<sup>M</sup> neurons in females constitutively expressing *fru*'s male-specific isoforms was determined.

Normally, Fru<sup>M</sup> proteins are expressed exclusively in males; however, *fru*<sup>M</sup> and *fru*<sup>Atra</sup> females constitutively express Fru<sup>M</sup> (Demir and Dickson, 2005). Therefore, the number of Fru<sup>M</sup>-expressing neurons in the Msg of *fru*<sup>M</sup> and *fru*<sup>Atra</sup> females was counted, to see if Fru<sup>M</sup> expression alone could abolish the observed difference in neuronal number in the Msg between the sexes. Figure 5.4 shows the number of Fru<sup>M</sup>-expressing neurons per hemisegment of the Msg in CS wild-type males ( $105.4 \pm 5.5$ ), *fru*<sup>M</sup> and *fru*<sup>Atra</sup> control males ( $102.3 \pm 7.2$  and  $110.3 \pm 4.8$ , respectively), and *fru*<sup>M</sup> and *fru*<sup>Atra</sup> females ( $75.3 \pm 3.7$  and  $73.1 \pm 4.6$ , respectively). The number of Fru<sup>M</sup>-expressing neurons in *fru*<sup>M</sup> and *fru*<sup>Atra</sup> females was significantly lower than in wild-type and control males ( $n=10$ ;  $p<0.05$ ). Thus Fru<sup>M</sup> expression alone does not create the sexually dimorphic population of neurons in the Msg, suggesting that another factor(s) may be required.

### 5.3.2.2 Is Dsx Required to Create a Sexually Dimorphic Mesothoracic Ganglion?

Dsx co-localizes with Fru<sup>M</sup> in the Msg, is required alongside Fru<sup>M</sup> to specify courtship song production, and controls the development of a sexually dimorphic population of neurons in the Abg (Taylor and Truman, 1992; Billeter *et al.*, 2006b). Thus, Dsx may play a critical role in the specification of the sexually dimorphic population of Fru<sup>M</sup>-expressing neurons in the Msg. To investigate whether Dsx is involved in specifying the sexually dimorphic population of Fru<sup>M</sup>-expressing neurons in this region, the number of Fru<sup>M</sup>-expressing neurons in the Msg was counted in *dsx* null mutant males.

Figure 5.4 shows the number of  $Fru^M$ -expressing neurons per hemisegment in the *Msg* of Canton S wild-type males, *dsx*<sup>1</sup> and *ln(3R)dsx*<sup>23</sup> control males, and *dsx* null mutant males. The number of  $Fru^M$ -expressing neurons per hemisegment in *dsx* null mutants is significantly lower compared to wild-type and control males ( $n=10$ ;  $p<0.05$ ). Interestingly, the difference in  $Fru^M$ -expressing neurons between wild-type males and *dsx* null mutant males was comparable to the difference in  $Fru^M$ -expressing neurons between wild-type males and *fru*<sup>M</sup> and *fru*<sup>Δtra</sup> females (~23 neurons). Therefore, *Dsx* expression is required for the specification of the sexually dimorphic population of  $Fru^M$ -expressing neurons in the *Msg*.



**Figure 5.4**-Number of  $Fru^M$ -Expressing Neurons per Hemisegment in the *Msg*. Mean number of nuclei expressing  $Fru^M$  per hemisegment ( $\pm$  s.d.) in the PrMs cluster of  $Fru^M$ -expressing neurons in 5- to 7-day-old adult flies (nomenclature as per Lee *et al.*, 2000). Mean number of nuclei per hemisegment calculated from 10 hemisegments per genotype. \* denotes a significant difference from wild-type CS and corresponding control males ( $p<0.05$ ). Presence or absence of  $Fru^M$ ,  $Dsx^M$  and  $Dsx^F$  are noted below each histogram bar.

### 5.3.2.3 Is *Dsx* Sufficient to Specify a Sexually Dimorphic Population of Neurons in the Mesothoracic Ganglion

*Dsx* expression is required to create a sexually dimorphic population of  $Fru^M$ -expressing neurons in the *Msg*. To determine whether *Dsx* expression alone was sufficient to specify this population of neurons, the *Msg* of females constitutively expressing the male isoform of *Dsx*,  $Dsx^M$ , was examined.

As females constitutively expressing  $Dsx^M$  do not express  $Fru^M$ , another marker must be found to determine the presence or absence of this sex-specific population of neurons. It was shown in 5.2.3 that  $Fru^M$  and  $Dsx$  are co-expressed in the *Msg* in approximately 17 neurons per hemisegment. Significantly,  $Dsx$  expression in the adult *Msg* is sexually dimorphic, where no  $Dsx$ -expressing neurons are present in the *Msg* of adult females (which lie in approximately the same position in *Msg* as the co-expressing neurons). Moreover, no  $Dsx$ -expressing neurons are present in the *Msg* of  $fru^M$  and  $fru^{Atra}$  females. Together, these results strongly suggest that the sexually dimorphic population of neurons in the *Msg* expresses both  $Fru^M$  and  $Dsx$ . As a result, the presence or absence of the sexually dimorphic population of neurons in the *Msg* can be determined by examining either the number of  $Dsx$ - or  $Fru^M$ -expressing neurons in this region.

Therefore, to determine whether the expression of  $Dsx^M$  alone is sufficient to specify the creation of the sexually dimorphic population of neurons in the *Msg*, the number of  $Dsx$ -expressing neurons in the *Msg* was counted. In wild-type males,  $Dsx$  is expressed in  $20.3 \pm 1.1$  neurons per hemisegment in the *Msg*, however, in females constitutively expressing  $Dsx^M$  (genotype  $dsx^{Swe}/dsx^{Df}$ ), no  $Dsx$ -expressing neurons were detected ( $n=7$ ). Thus the constitutive expression of  $Dsx^M$  in females is not sufficient to specify the creation of the sexually dimorphic population of neurons. How then is this sexually dimorphic population of neurons in the *Msg* created?

#### **5.3.2.4 Both $Dsx^M$ and $Fru^M$ Are Required to Create a Sexually Dimorphic Population of Neurons in the Mesothoracic Ganglion**

$Dsx$  and  $Fru^M$  co-localize in the *Msg*, and neither  $Fru^M$  nor  $Dsx^M$  alone can specify the creation of the sexually dimorphic population of  $Fru^M$  neurons in the *Msg* of females. In the *Abg*, both  $Fru^M$  and  $Dsx$  are required for the development of a male-specific cluster of serotonergic neurons in the *Abg* (Billeter *et al.*, 2006b). To determine whether the same is true in the *Msg*, the number of  $Fru^M$ -expressing neurons in females constitutively expressing both  $Fru^M$  and  $Dsx^M$  was examined.

*tra* null mutant females express both  $Fru^M$  and  $Dsx^M$  (Hoshijima *et al.*, 1991; Ryner *et al.*, 1996). When the number of  $Fru^M$ -expressing neurons in the *Msg* of these females was tabulated, the number of  $Fru^M$ -expressing neurons was not

significantly different from the number of Fru<sup>M</sup>-expressing neurons in wild-type males (Figure 5.4) (n=10; p>0.05). Therefore, a wild-type number of Fru<sup>M</sup>-expressing neurons in the Msg was observed only when both Fru<sup>M</sup> and Dsx<sup>M</sup> were present, demonstrating a previously unrecognized requirement for Dsx<sup>M</sup> in the specification of a population of Fru<sup>M</sup> neurons. Moreover, this result provides further evidence to support the hypothesis that in regions of co-localization, Fru<sup>M</sup> and Dsx act together to specify a sexually dimorphic neuronal substrate involved in the sex-specific performance of courtship behaviours.

## **5.4 Does the Sexually Dimorphic Population of Fru<sup>M</sup>-Expressing Neurons Include Any of the mnDFMs?**

In the previous section, a sexually dimorphic population of Fru<sup>M</sup>-expressing neurons was identified in the Msg. Previous studies have shown that the cell bodies of many mnDFMs lie in the Msg (Trimarchi and Schneiderman, 1994), raising the possibility that the neurons innervating the DFMs are included in this sexually dimorphic population of neurons.

### **5.4.1 Are Any DFMs Innervated by fru Neurons?**

The sexually dimorphic population of neurons in the Msg consists of a subset of the *fru*-expressing neurons in this region. Therefore, *fru*<sup>GAL4</sup> was used to drive the GAL4-responsive membrane-bound mCD8::GFP reporter construct to determine whether any of the DFMs were innervated by *fru* neurons. Indeed, the Type I synaptic terminals of one of the DFMs, B3/B4, was innervated by a *fru* neuron (Figure 5.5).

### **5.4.2 Is This Innervation Sexually Dimorphic?**

Given that the number of *fru* neurons in the Msg is sexually dimorphic, and that one of the DFMs is innervated by a *fru* neuron, mnB3/B4 was examined to determine if the neurons were *fru*<sup>GAL4</sup>-positive in both sexes. Previous studies have shown that at the embryonic NMJ, when a muscle is deprived of its cognate neuron, motor neurons in the immediate proximity to the muscle will often form synapses with the neuron-deprived muscle (Chang and Keshishian, 1996). If mnB3/B4 is part of the sexually dimorphic population of neurons in the Msg, an

equivalent neuron may not exist in both sexes, ultimately leading to the sexually dimorphic innervation of one of the DFMs.

Figure 5.5 shows the innervation of B3/B4 in both males and females by a *fru*<sup>GAL4</sup>-positive neuron. Thus it seems that B3/B4 are innervated by equivalent neurons in both males and females, and that mnB3/B4 is not included in the sexually dimorphic population of *fru* neurons in the Msg.

#### **5.4.3 Is This Innervation Affected by *dsx* Mutations?**

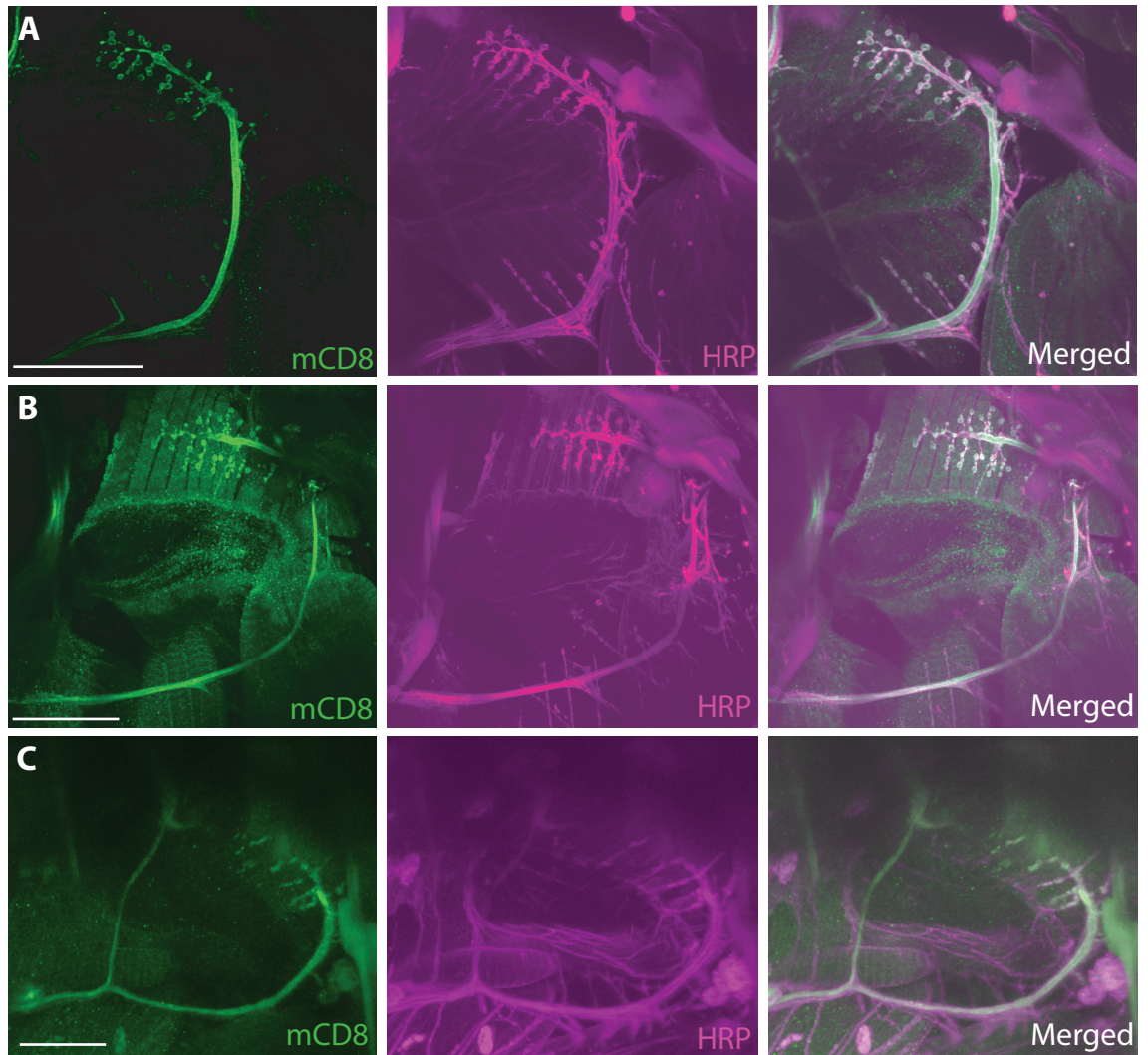
Null mutations in *dsx* also affect the sexually dimorphic population of *fru* neurons in the Msg; in addition, *dsx* mutant males have aberrant courtship song. Therefore, in order to determine whether mnB3/B4 is affected by the reduction in *fru*-expressing neurons in the Msg in *dsx* mutants, mnB3/B4 was examined to determine if the neurons remained *fru*<sup>GAL4</sup>-positive in *dsx* null mutant males.

Figure 5.5 shows that mnB3/B4 was *fru*<sup>GAL4</sup>-positive in both wild-type and *dsx* null mutant males, demonstrating that the *fru* neuron innervating B4/B4 is not included in the population of *fru* neurons that is affected by mutations in *dsx*. These results, together with the data from Chapter 4, provide solid evidence that both the sexually dimorphic production of song, and the song defects of *fru* and *dsx* mutant males, are not a result of sex-specific or aberrant innervation of the DFMs. Instead, the sexually dimorphic population of Fru<sup>M</sup> neurons in the Msg found in this chapter may form part of a male-specific neural network in the CNS responsible for controlling the production of courtship song.

### **5.5 What is the Mechanism by which a Sexually Dimorphic Mesothoracic Ganglion is Created?**

Both Fru<sup>M</sup> and Dsx have been shown to be involved in creating sexually dimorphic neuronal populations in the CNS. Fru<sup>M</sup> expression in a specific cluster of neurons in the brain prevents programmed cell death in this region (Kimura *et al.*, 2005), whereas Dsx is key to prolonging neuroblast divisions in the male Abg (Taylor and Truman, 1992). In the Msg, Fru<sup>M</sup> and Dsx<sup>M</sup> must both be present in order to specify a sexually dimorphic population of neurons, however, it is not clear whether this population of neurons is created by sex-specifically preventing cell

death, sex-specifically prolonging neuroblast divisions, or some combination of both. Therefore, the mechanism by which the sexually dimorphic population in the *Msg* of neurons is generated was investigated in more detail.



**Figure 5.5-***fru*<sup>GAL4</sup> generates expression in a single mnDFM. *fru*<sup>GAL4</sup> was used to drive expression of the mouse lymphocyte (transmembrane) marker CD8 and green fluorescent fusion protein (UAS-mCD8::GFP), and detected by anti-mCD8 labelling (mCD8; green). Anti-HRP conjugated to Cy3 (HRP; purple) was used to reveal the neuronal projections of mnB3/B4 (purple) (Jan and Jan, 1982). *fru*<sup>GAL4</sup> drives expression in mnB3/B4 in both 5- to 7-day-old males (A), females (B), and *dsx* mutant males (C; genotype *In(3R)dsx*<sup>23</sup>/*Df(3R)dsx*<sup>15</sup>). DFM nomenclature as per Ewing (1979). (A-C) Maximum Z-projections of confocal images are shown. Scale bar = 50μm.



### 5.5.1 Cell Death

Apoptosis in *Drosophila* requires three genes: *rpr*, *hid* and *grim* (White *et al.*, 1994; Grether *et al.*, 1995; Chen *et al.*, 1996; White *et al.*, 1996). All three genes activate a caspase pathway to promote cell death (Hay *et al.*, 1994; Bump *et al.*, 1995; Grether *et al.*, 1995; Chen *et al.*, 1996; White *et al.*, 1996; McCarthy and Dixit, 1998; Haining *et al.*, 1999), and have been shown to play important roles during the development of the CNS. *rpr*, *hid* and *grim* are located in tandem along the chromosome, and are all removed by a single deficiency, *Df(3L)H99* (White *et al.*, 1994). The effects of individual genes are generally examined by using specific deficiencies *in trans* to *Df(3L)H99*; however, many alleles of *grim* or *hid* result in embryonic lethality, and the emergence of adult flies is very rare (Grether *et al.*, 1995; Chen *et al.*, 1996; Peterson *et al.*, 2002).

Previously, Kimura *et al.* (2005) showed that programmed cell death was responsible for the death of a cluster of neurons in the female brain, creating a sexually dimorphic population of neurons in this region. In *rpr* mutant females, a significant increase in the number of neurons in this cluster was observed. In *rpr* mutant males, however, no increase in the number of neurons in this region was found, and Kimura *et al.* (2005) concluded that sex-specific cell death was the sole determinant of the sexually dimorphic neuronal population in this region of the brain.

Therefore, to determine the effect of *rpr*-mediated cell death on the sexually dimorphic population of neurons in the *Msg*, *Dsx*-expressing neurons were counted in *rpr* mutant males and females. The number of *Dsx*-expressing neurons in *rpr* mutant males was not significantly different from wild-type males ( $n=10$ ;  $p>0.05$ ). Moreover, in *rpr* mutant females, no *Dsx*-expressing neurons were observed, suggesting that *rpr*-mediated cell death does not contribute to the creation of the sex-specific population of neurons in the *Msg*. It is possible that *hid*- or *grim*-induced apoptosis could be responsible for the sex-specific death of the neurons in the *Msg*; however, as mutations in either gene are lethal, it is very difficult to study the effects of these genes on the creation of a sex-specific *Msg* without resorting to mosaic analysis.

### 5.5.2 Neuronal Proliferation

In *Drosophila*, neuroblasts divide asymmetrically, to generate two daughter cells: a larger cell, which remains a neuroblast, and a smaller cell, called a ganglion mother cell (GMC), which divides symmetrically to produce two postmitotic neurons (reviewed by Jan and Jan, 2001; Chia and Yang, 2002; Betschinger and Knoblich, 2004). In the Abg, it has been shown that *dsx* is responsible for prolonging neuroblast divisions in males, resulting in more neurons in the Abg (Truman and Bate, 1988; Taylor and Truman, 1992). Given that  $Dsx^M$  is required for the specification of a male-specific population of  $Fru^M$ -expressing neurons in the Msg, *Dsx* may play a similar role in the Msg. Typically, BrdU incorporation is used to determine the number of actively dividing neuroblasts in the CNS; however, some regions of the CNS, including the Msg, contain so many actively dividing neuroblasts that distinguishing individual cells is difficult, and makes comparisons between the sexes virtually impossible (Taylor and Truman, 1992). Thus, the contribution of neuronal proliferation to the creation of a sexually dimorphic population of  $Fru^M$ -expressing neurons in the Msg could not be determined using BrdU labelling.

## 5.6 Discussion

In this chapter, a detailed examination of the Msg was performed, to identify any sex-specific characteristics that could contribute to the sexually dimorphic nature of courtship song production. Furthermore, the role of sex determination genes *fru* and *dsx* in creating these differences was examined. The major findings and their implications are discussed below.

### 5.6.1 Co-Expression of $Fru^M$ and *Dsx* in the CNS

In the CNS, sexually dimorphic neuronal populations have been reported (Truman and Bate, 1988; Taylor and Truman, 1992; Kimura *et al.*, 2005; Billeter *et al.*, 2006b); however, the combined effects of both *fru* and *dsx* in creating these differences in the CNS had not been investigated in detail. This chapter revealed the co-expression of  $Fru^M$  and *Dsx* in three distinct regions of the CNS: the posterior brain, the Abg, and the Msg. Given the known roles of both *fru* and *dsx* in creating sexually dimorphic neuronal populations elsewhere in the CNS

(Taylor and Truman, 1992; Kimura *et al.*, 2005), it seems likely that sexually dimorphic developmental mechanisms may be operating in the regions in which Fru<sup>M</sup> and Dsx are co-expressed.

Previously, Lee *et al.* (2002) observed that fewer Dsx-expressing neurons were present in the posterior brain of adult females. Since, other studies have shown that a male-specific cluster of neurons is also present in the Abg (Truman and Bate, 1988; Taylor and Truman, 1992; Billeter *et al.*, 2006b), and this chapter has shown that a sexually dimorphic population of *fru* neurons is also present in the Msg. Thus, a consequence of Fru<sup>M</sup> and Dsx co-expression in the CNS seems to be the creation of sex-specific populations of neurons, a hypothesis supported by the finding by Lee *et al.* (2000) that *fru*'s P1 transcripts are sex-specifically expressed in only three regions of the CNS: the posterior brain, the Msg and the Abg. Given that all three regions have also been reported to be essential for the performance of male-specific behaviours such as tapping, wing extension, licking, courtship song, attempted copulation and copulation (Hall, 1979; von Schilcher and Hall, 1979; Ferveur and Greenspan, 1998), these populations of neurons may form individual parts of a male-specific neural network responsible for the control of male courtship behaviour.

### **5.6.2 The Neurobiological Basis of Courtship Song**

Overall, a critical aim of this thesis is to investigate the neurobiological basis for courtship song production. The Msg is thought to contain the neural foci for courtship song (von Schilcher and Hall, 1979), and in this chapter, a sexually dimorphic population of Fru<sup>M</sup> neurons was found in the Msg. This population of neurons consists of approximately 23 neurons per hemisegment in the Msg, and its creation requires the presence of both Fru<sup>M</sup> and Dsx<sup>M</sup>. These findings are significant, as they show a novel requirement for Dsx<sup>M</sup> in the specification of a population of Fru<sup>M</sup> neurons. Moreover, it is another example of *fru* and *dsx* acting together to determine a sexually dimorphic cluster of neurons in a region strongly implicated in a male-specific behaviour (Billeter *et al.*, 2006b).

How then does a sexually dimorphic cluster of neurons influence the production of courtship song? The presence of this cluster of approximately 23 neurons per hemisegment in the Msg is correlated with the ability to produce wild-type

courtship song. Results from both the previous chapter and this chapter show that these 23 neurons are not sexually dimorphic motor neurons that directly innervate the DFM. Instead, they may be interneurons, forming a vital link between regions of the brain receiving stimulation from the opposite sex (either gustatory, olfactory or otherwise), and the motor neurons responsible for the motor output required for song production. Although the signals to initiate courtship and wing extension and song may be received by *fru*<sup>M</sup> and *fru*<sup>Δtra</sup> females and *dsx* mutant males, the absence of these sex-specific neurons in these individuals could mean that these signals are not communicated to the areas producing the motor output.

### ***5.6.3 Uncovering the Mechanism Responsible for Creating Sexually Dimorphic Msg***

Although a sexually dimorphic population of neurons has been found in the Msg, it is of critical importance to discover the mechanism by which the dimorphism is created. Previously, Fru<sup>M</sup> has been shown to create sexually dimorphic neuronal populations by ‘protecting’ neurons against programmed cell death. Dsx, on the other hand, acts on neuroblasts, prolonging their divisions, and increasing the number of neurons in a given sex. It was shown in section 5.5.1 that it is unlikely that *rpr*-mediated cell death alone is responsible for the observed dimorphism in the Msg. Given that both Fru<sup>M</sup> and Dsx<sup>M</sup> are required to obtain a full complement of neurons in the Msg, perhaps the dimorphic population of *fru* neurons is created by the action of Dsx<sup>M</sup> to prolong neuroblast divisions of specific cells in the Msg, and then Fru<sup>M</sup> protects the neurons from programmed cell death.

### ***5.6.4 Conclusions***

In this chapter, the co-expression of Fru<sup>M</sup> and Dsx in three distinct regions of the CNS was shown. In addition, a sexually dimorphic population of neurons in one of these regions, the Msg, was identified, and its creation was found to rely on the expression of both Dsx<sup>M</sup> and Fru<sup>M</sup>. Given the importance of the Msg to song production, it is intriguing that the presence or absence of this population of neurons is correlated with the ability to produce courtship song. Moreover, the creation of this population of neurons, like courtship song production, is

dependent on both  $Fru^M$  and  $Dsx^M$ . The precise role of this population of neurons in courtship song remains unknown, and it remains unclear how the absence of this sexually dimorphic population in *dsx* mutant males and *fru<sup>M</sup>* and *fru<sup>Δtra</sup>* females can cause such striking defects in courtship song. Therefore, genetic tools are required to study the anatomy, development and function of this group of neurons, to gain an understanding of how this group of neurons modulates the production of courtship song.

## **6 Generation of a *dsx*-GAL4 Promoter Fusion Construct to Characterize *dsx* Neurons**

## 6.1 Introduction

In previous chapters, a requirement for both Fru<sup>M</sup> and Dsx<sup>M</sup> in the specification of courtship song and the creation of a sexually dimorphic population of neurons in the *Msg* was shown. The presence of these sex-specific neurons is linked to the ability to perform courtship song; however, other properties of these neurons remain unknown. Therefore, a genetic tool is required to anatomically and functionally characterize these neurons, and to investigate the roles of Fru<sup>M</sup> and Dsx<sup>M</sup> in specifying these properties.

Given that the sexually dimorphic population of neurons in the *Msg* expresses both Fru<sup>M</sup> and Dsx<sup>M</sup>, a genetic tool to visualize and manipulate either *fru*- or *dsx*-expressing neurons would allow the characteristics of these neurons to be investigated. In the CNS, Fru<sup>M</sup> is expressed in approximately 2000 neurons (Lee *et al.*, 2000; Billeter and Goodwin, 2004), whereas Dsx is expressed in approximately 600 neurons (Lee *et al.*, 2002). In the *Msg*, Fru<sup>M</sup> is expressed in ~200 neurons, whereas Dsx is expressed in only ~40 neurons. A genetic tool which directs expression in all *fru* neurons, called *fru*<sup>GAL4</sup>, drives expression in all 200 neurons in the *Msg*, making the isolation, manipulation and description of only the 23 sex-specific neurons per hemisegment (~46 total in the *Msg*) more difficult. On the other hand, a genetic tool directing expression in *dsx* neurons in the *Msg* would allow the properties of this sex-specific population of neurons to be examined in isolation. Therefore, this chapter will describe the construction of a *dsx* promoter-GAL4 fusion transgene, and document the spatial and temporal patterns of expression generated by this transgene.

## 6.2 Generation of Promoter Fusion Constructs

### Advantages and Disadvantages

GAL4 is a yeast regulatory protein with specific DNA binding sites called UAS<sub>G</sub> (galactose upstream activating sequences) (Guarente *et al.*, 1982; Yocum *et al.*, 1984; West *et al.*, 1984; Johnston and Davis, 1984; Bram and Kornberg, 1985; Giniger *et al.*, 1985). Fischer *et al.* (1988) first reported that the tissue-specific expression of GAL4 could activate the expression of a reporter gene fused downstream to UAS sites in these same tissues in *Drosophila*. This result showed

that the spatial and temporal expression patterns of specific genes could be investigated by fusing the promoter from the gene of interest to GAL4, and then examining the expression of a reporter fused to the UAS sites. In addition, the promoter-GAL4 fusion lines could be used to ‘drive’ the expression of any transgene placed downstream of the UAS sites. Thus, the UAS-GAL4 system allows the anatomy, development and function of any subset of cells to be examined, provided an appropriate promoter is chosen.

Despite its many advantages, there are a number of disadvantages of using the UAS-GAL4 system. First, the introduction of the promoter-GAL4 fusion and the UAS-reporter/transgene fusion constructs into the genome requires the use of *P*-element transformation, where the transgenic constructs are inserted at random into the genome (Rubin and Spradling, 1983). The chromatin configuration at the site of UAS-reporter/transgene insertion can have strong effects on the expression of the reporter, inducing expression even in the absence of the GAL4 (Spradling and Rubin, 1983). Likewise, these *cis* regulatory elements around the site of promoter-GAL4 fusion insertion can inappropriately interact with the promoter sequence in the transgene, and GAL4 expression may not precisely reflect the expression dictated by the promoter (Spradling and Rubin, 1983). To avoid these ‘position effects’, multiple independent insertion lines are typically screened to minimize line-to-line variation. This screening is especially important in choosing UAS-reporter/transgene lines, as lines generating expression in the absence of GAL4 can give misleading data about the spatial and temporal expression of the gene under examination. Thus, new *P*-element transformation vectors have been published which contain genetic elements to counteract these ‘position effects’ and reduce the line-to-line variation (Barolo *et al.*, 2000).

### **6.2.1 Vectors**

A new type of *Drosophila* transformation vector has been developed to reduce the effect of insertion site on the expression driven by the promoter-GAL4 fusion transgene (Barolo *et al.*, 2000). These new transformation vectors are called Pelican vectors (*P*-element *lacZ*/GFP-insulated *CaSpeR* enhancer vectors), and are based on pCaSpeR-*lacZ* (Margolis *et al.*, 1994), which is derived from pCaSpeR (Pirrotta, 1988). The Pelican vectors have further improvements on



earlier reporter vectors, the most important of which is two copies of the insulator sequence from the *gypsy* transposon which flank the region containing the reporter (Barolo *et al.*, 2000). These insulators each contain 14 binding sites for the suppressor-of-Hairy-wing (su(Hw)) protein (Spana *et al.*, 1988; Geyer and Corces, 1992), and have been shown to reduce the effects of chromatin configuration when flanking a marker transgene in *Drosophila* (Patton *et al.*, 1992; Roseman *et al.*, 1993; Roseman *et al.*, 1995). Moreover, the insulator is capable of blocking some enhancers and silencers when placed in between the enhancer/silencer and a promoter (Cai and Levine, 1995; Barolo and Levine, 1997). A final improvement of the Pelican vectors on earlier reporter vectors is the addition of a new multiple cloning site, which adds between fourteen and nineteen unique restriction sites to the *EcoRI*, *BamHI* and *KpnI* sites of pCaSpeR- $\beta$ -Gal (Barolo *et al.*, 2000). Therefore, promoter fragments from *dsx* will replace the reporter-GFP/*lacZ* inserts in the Pelican *P*-element transformation vector called pStinger. This replacement will minimize chromatin effects on the *dsx* promoter, reducing line-to-line variation in the expression of GAL4. This approach has been previously shown to be successful by Billeter and Goodwin (2004), where *fru* promoter fragments were inserted into pStinger and integrated randomly into the genome by *P*-element transformation (Rubin and Spradling, 1983). Independent insertions of the *fru* promoter-GAL4 fusion construct into the genome generated consistent patterns of expression (Billeter, 2003). Moreover, the expression driven by these promoter-GAL4 lines was congruent with subsets of *fru*-expressing neurons (Billeter and Goodwin, 2004).

## **6.2.2 *dsx*(15)-GAL4 Promoter Fusion Construction**

### **6.2.2.1 Selection of *dsx* Genomic Region**

Long-distance interactions between enhancers and promoters have been documented in *Drosophila* (Blackman *et al.*, 1991; Pirota, 1995; reviewed by Sipos and Gyurkovics, 2005). For example, polycomb response elements (PRE) are capable of controlling the expression of the *Ultrabithorax* (*Ubx*) gene from tens of kilobases upstream (Pirota, 1995). Similarly, the expression of the *decapentaplegic* (*dpp*) gene is regulated by enhancers spanning nearly 30 kb downstream of the *dpp* coding region (Blackman *et al.*, 1991). Thus sequences

containing regulatory information for a given gene can be found at some distance upstream or downstream of the coding region.

The *dsx* locus spans approximately 43 kb, from cytogenetic location 84E5 to 84E6 (Figure 6.1). The distance between the predicted RNA start site and the next upstream gene (5' to *dsx*) is approximately 15 kb, and the distance between the first and the second coding exons is approximately 24 kb (Baker and Wolfner, 1988; Burtis and Baker, 1989). Enhancers or repressors controlling the expression of *dsx* may be found in either of these regions, or even further up- or downstream. Given that *P*-element transformation efficiency depends on the size of the transgenic construct, where the expected germ-line transformation frequency for constructs of 15 kb or less is approximately 5-14% of the fertile injected embryos (Spradling, 1986), the 5' region from the translation start site to the gene located upstream of *dsx* will be inserted into the pStinger *P*-element transformation vector to make the *dsx* promoter-GAL4 fusion transgene (Figure 6.2). Although a 43 kb cosmid was successfully transformed by Haenlin *et al.* (1985), the transformation frequency was less than 1%. Thus, it may be theoretically possible to insert a larger *dsx* promoter fragment into the pStinger vector; however, the very low efficiency of transforming such a large transgene may ultimately reduce the likelihood of recovering an insertion line capable of directing expression.

#### **6.2.2.2 Isolation of *dsx* Promoter Fragments and Construction of *dsx(15)*-GAL4 Transgene**

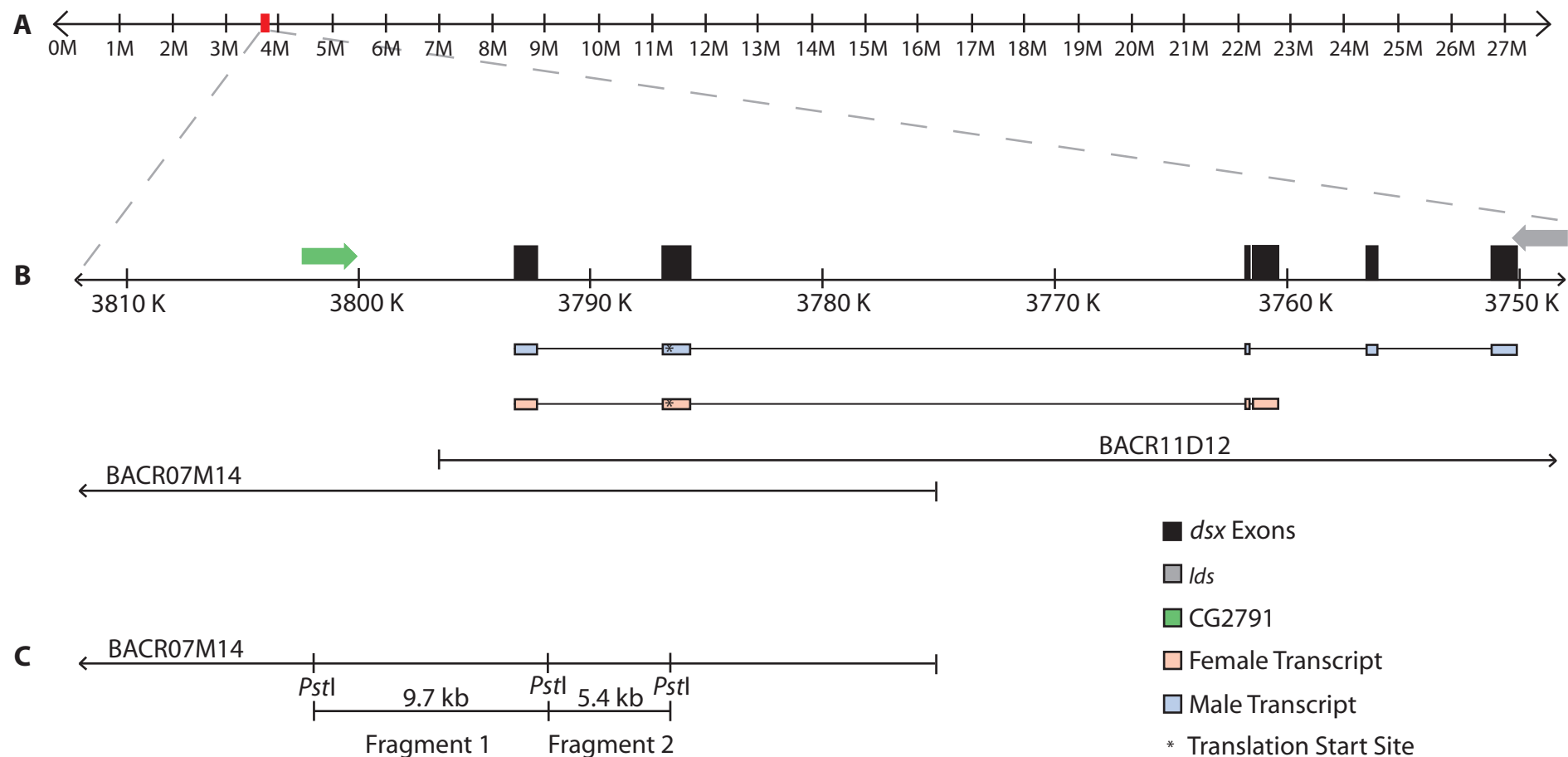
Two bacterial artificial chromosomes (BAC), BACR07M14 (84F-84F) and BACR11D12 (84D-84E), have inserts overlapping the *dsx* locus (Figure 6.1). BACR07M14 was chosen to isolate the desired promoter region of *dsx*, as it contains the 5' regulatory region upstream of the *dsx* transcriptional and translational start sites.

Restriction sites in the sequence of BACR07M14 were predicted using Mac Vector 7.2.2 (Oxford Molecular, Invitrogen), and selected based on ease of fragment insertion into the subcloning vector pZErO-2 (Invitrogen). pZErO-2 contains a gene in its polylinker that encodes a product lethal to *E. coli*. This facilitates the cloning of large fragments, as the *E. coli* can only survive to amplify the plasmid if genomic fragments have been successfully inserted into the polylinker. To

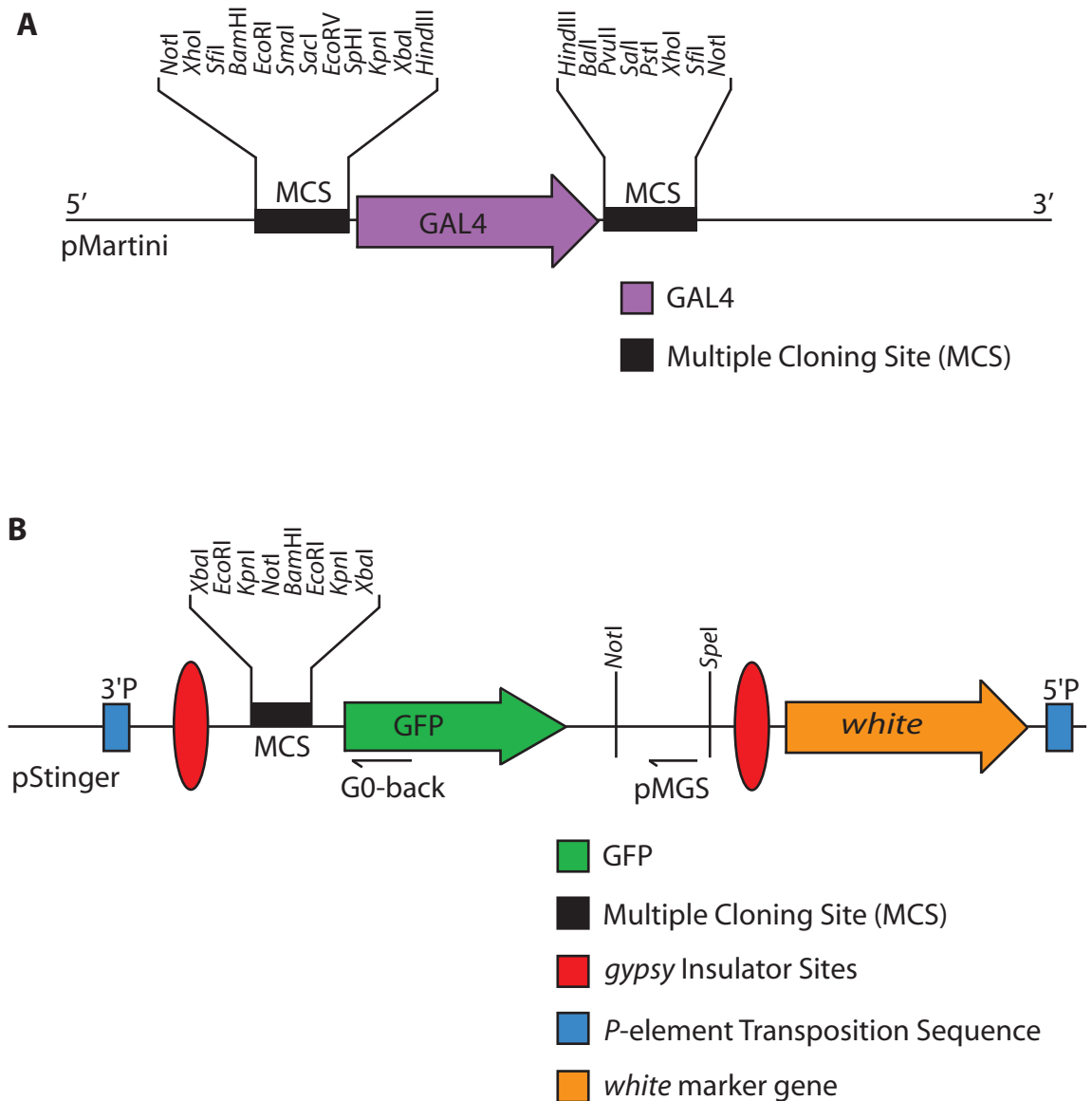
further increase the probability of obtaining recombinant plasmids containing large genomic inserts, the plasmids were transformed into *E. coli* by electroporation. Unlike other methods of transformation, electroporation is less biased towards the transformation of small plasmids, allowing the successful transformation of plasmids upwards of >300 kb (Hanahan *et al.*, 1991).

The cloning strategy to make a *dsx* promoter-GAL4 fusion is shown in Figure 6.3. First, BACR07M14 was digested with *Pst*I, and the digest was run on 0.8% agarose gels to separate the fragments. The agarose gel was stained with crystal violet and visualized on a light box to eliminate any damaging exposure to UV. Two bands corresponding to fragments of the desired sizes, 9.7 kb (Fragment 1) and 5.4 kb (Fragment 2), were excised from the agarose gels and purified using the QIAEXII large fragment purification kit (QIAGEN). Each fragment was then ligated into the *Pst*I-digested pZER0-2 sub-cloning vector. Large numbers of colonies (~70 colonies per fragment) were screened using the ‘cracking’ method to find the recombinant plasmids containing the desired *dsx* promoter fragments.

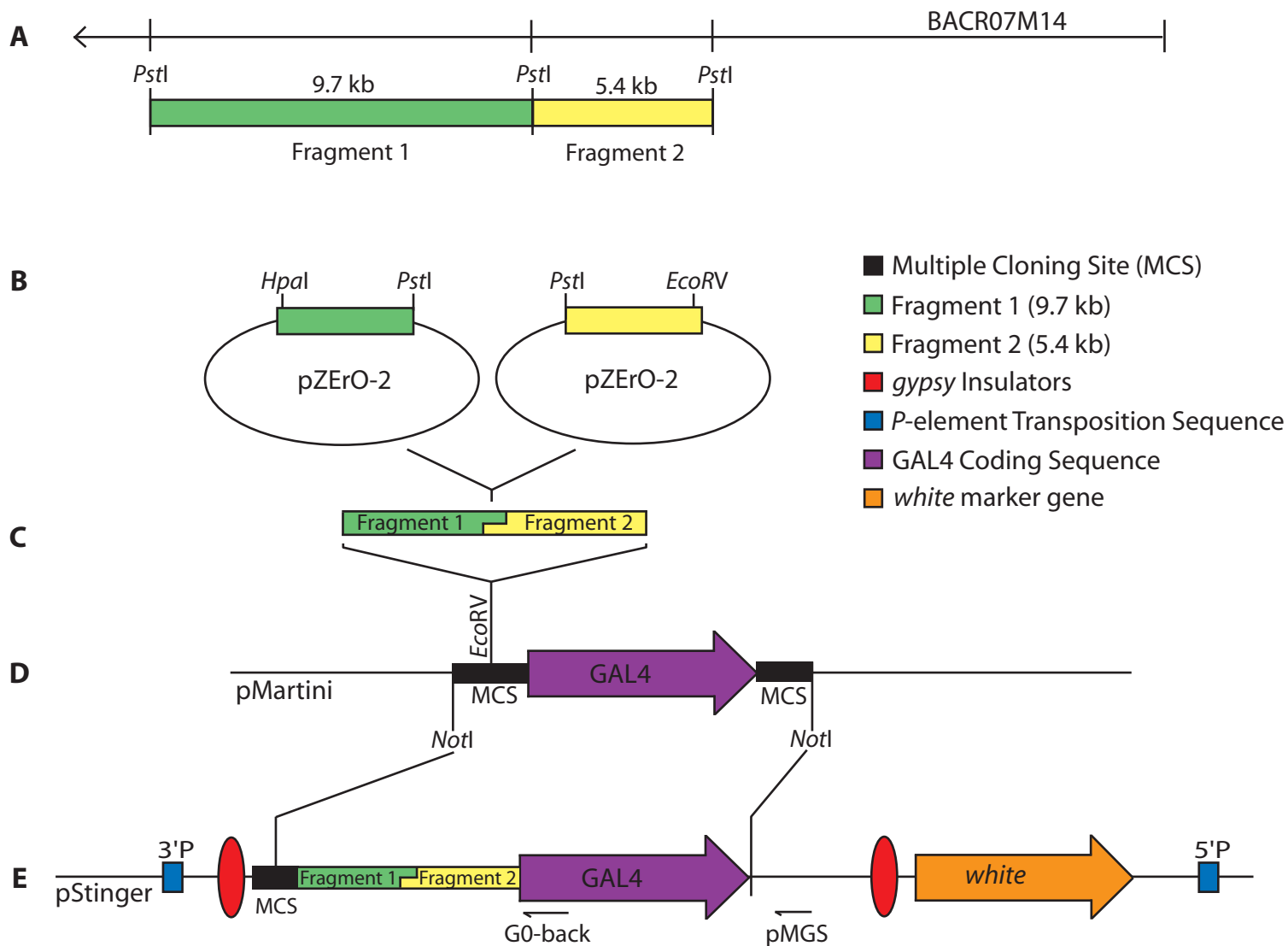
Next, the Fragment 1-containing pZER0-2 was digested with *Hpa*I and *Pst*I, and the Fragment 2-containing pZER0-2 was digested with *Pst*I and *Eco*RV, and fragments of 9.6 kb and 5.3 kb were isolated and purified as described above. Prior to ligation into the GAL4-containing pMartini vector (Figure 6.2 and 6.3) (Billeter, 2003), the purified fragments were mixed in a 1:1 ratio, heated to 50°C, and then cooled slowly to room temperature, to anneal the complementary overhanging ends generated by the *Pst*I digest. This blunt-ended fragment of ~15 kb was then ligated into the *Eco*RV-digested pMartini-GAL4. The orientation of the promoter fragment inserted into pMartini-GAL4 was verified by restriction digest. Finally, the *dsx* promoter fragment fused to the GAL4 from pMartini-GAL4 was excised from pMartini-GAL4 using the *Not*I restriction sites, and the promoter-GAL4 fragment was inserted into the *Not*I-digested pStinger vector (Figure 6.2 and 6.3) (Barolo *et al.*, 2000; Billeter, 2003; Billeter and Goodwin, 2004) to generate the *dsx* promoter-GAL4 fusion construct *dsx*(15)-GAL4.



**Figure 6.1-** Overview of *dsx* Genomic Locus and Overlapping BACs. (A) Map of the right arm of the third chromosome. Position of the *dsx* genomic locus indicated by the solid red box. M = megabases. (B) Zoomed-in view of the *dsx* genomic locus showing the exons in black, and the sex-specific transcripts in pink and blue below. Neighbouring genes *lds* and CG2791 are also shown. The BACs BACR07M14 and BACR11D12 span the entire *dsx* locus. (C) Restriction sites on BACR07M14 used to isolate the *dsx* promoter fragments are indicated.



**Figure 6.2-*dsx*(15)-GAL4 Cloning Vectors pStinger and pMartini.** (A) Diagram of pMartini-GAL4 plasmid vector (Billeter, unpub.). Multiple cloning site/polylinker indicated by 'MCS', and the restriction enzymes contained in the polylinker are shown. The coding sequence of GAL4 is flanked by the two polylinkers. *dsx* promoter fragments were cloned into the MCS at the 5' end of the GAL4 coding sequence. (B) Diagram of pStinger. Blue boxes represent *P*-element transposition sequences, the black box (along with two restriction sites indicated downstream) represents the polylinker and restriction sites used to clone the *dsx* promoter sequence into the insulated vector. Red boxes represent the *gypsy* insulators, and the *white* gene marker used to select transformants is indicated in orange.



**Figure 6.3-** Cloning Strategy for *dsx(15)*-GAL4. Two fragments were excised from BACR07M14 using the restriction enzyme *PstI* (A). The fragments, 9.7 and 5.4 kb, respectively, were then ligated into the *PstI*-digested pZER0-2 vector (B). Fragment 1 was removed from pZER0-2 using *HpaI* and *PstI* restriction sites; Fragment 2 was removed using *PstI* and *EcoRV* sites. The fragments were annealed by mixing the two in a 1:1 ratio, heated to 50°C, and allowed to cool to room temperature (C). This blunt-ended annealed fragment was ligated into an *EcoRV*-digested pMartini-GAL4 vector (Billeter, unpub.) (D). Finally, *NotI* was used to excise the *dsx* promoter-GAL4 fusion and the large fragment was inserted into a *NotI*-digested pStinger vector (E; pStinger is as described in Figure 6.2).

## 6.3 Generation and Chromosomal Mapping of Transformant Flies

### 6.3.1 Identification and Chromosomal Mapping of Transformants

The final *dsx(15)*-GAL4 promoter-GAL4 fusion transgene was approximately 30 kb in size, and was microinjected into *w<sup>1118</sup>* embryos by EMBL *Drosophila* Injection Service (Heidelberg). Following the injection, approximately 300 larvae were returned, and 14 transformants were recovered. The chromosome of insertion for each transgenic line was determined (Table 6.1).

### 6.3.2 Identification of Functional *dsx(15)*-GAL4 Transformants

#### 6.3.2.1 X-gal Screening of Transformants

Once transgenic flies containing the *dsx* promoter-GAL4 fusion were recovered, it was important to determine whether these transgenes were capable of driving the expression of a reporter gene. The ultimate goal of the *dsx* promoter-GAL4 fusion was to construct a genetic tool allowing the expression of a variety of reporters and transgene in *dsx*-expressing neurons, to gain a better understanding of their structure and function. Thus, males or females from each of the 14 independent insertion lines were crossed to a line of transgenic flies containing the *lacZ* gene under the control of the GAL4-responsive UAS enhancer. The progeny from this cross were dissected and placed in X-Gal staining solution, then incubated to allow the reaction to proceed and the expression pattern to be visualized. The results from these dissections are shown in Table 6.1.

Transformant	Chromosome	<i>lacZ</i> Expression
1	4	No
2	3	No
3	2	No
4	3	No
5	3	No
6	X	Yes
7	4	No
8	3	No
9	2	No
10	3	No

Transformant	Chromosome	<i>lacZ</i> Expression
11	3	No
12	X	No
13	3	No
14	2	No

**Table 6.1-*dsx(15)*-GAL4 Transformant Lines. Fourteen independent insertion lines of the *dsx(15)*-GAL4 transgene were obtained, and mapped to a chromosome. All insertion lines were then crossed to a *lacZ* reporter gene downstream of the GAL4-responsive UAS element, and the progeny were scored for expression.**

Of the fourteen independent insertion lines of the *dsx(15)*-GAL4 transgene, a single line, #6, was found to drive the expression of a reporter. This line will now be referred as *dsx(15)*-GAL4, and the spatial and temporal patterns of expression driven by this line will be the focus of the remainder of this chapter.

## 6.4 Characterization of *dsx(15)*-GAL4

The primary aim of this chapter was to construct a genetic tool to investigate the anatomy and function of *dsx*-expressing neurons. However, given the importance of *dsx* in determining the sex of non-neuronal tissues (reviewed by Cline and Meyer, 1996; Christiansen *et al.*, 2002; Billeter *et al.*, 2006a), the expression driven by *dsx(15)*-GAL4 in adult tissues outwith the CNS will also be documented.

### 6.4.1 Non-Neuronal Expression

#### 6.4.1.1 Adult Expression

Figure 6.4 shows the *lacZ* expression driven by *dsx(15)*-GAL4 in the adult. The expression driven by *dsx(15)*-GAL4 was fairly widespread, and found in a number of tissues implicated in sex-specific functions, such as the internal reproductive organs and sensory structures.

#### Sensory Structures

Strong *lacZ* expression was detected in sensory structures such as the maxillary palps and the antennae (Figure 6.4). This expression was not detected in the control X-gal staining of the UAS-*lacZ* line alone. The expression of *lacZ* in these



structures suggests that *dsx* plays a role in the sexually dimorphic development and function of these regions. Indeed, the maxillary palps and antennae have been implicated in the detection of sex-specific pheromones during courtship (reviewed by Stocker, 2001; Greenspan and Ferveur, 2000; Billeter *et al.*, 2006a). Thus *dsx(15)*-GAL4 may be an excellent tool to examine the sex-specific development and physiology underlying the sexually dimorphic functions of these sensory structures. Another sexually dimorphic sensory structure in which *dsx* has been implicated in sex-specific function is a set of male-specific gustatory bristles on the foreleg. *dsx* is required for the expression of a gustatory receptor called *Gr68a* in the neurons in these male-specific bristles, a receptor thought to be involved in pheromone detection (Bray and Amrein, 2003). Unfortunately, strong expression in the muscles of the legs made it impossible to ascertain whether *lacZ* expression was present in the male-specific gustatory bristles on the foreleg.

### Internal Reproductive Organs and Genitalia

Strong *lacZ* expression was also detected in the internal reproductive organs of both sexes. Again, this expression was not detected in the control X-gal staining of the UAS-*lacZ* line alone. Expression in the male reproductive organs was observed in the ejaculatory bulb, the ejaculatory duct, the seminal vesicles, the testicular duct, the vas deferens and the apical tip of the accessory glands. These organs are critical for male fertility, supplying the sperm required to fertilize the females' eggs, as well as seminal fluids that increase ovulation and decrease receptivity in females (reviewed by Kubli, 2003). In the female internal reproductive organs, expression was detected in the common and lateral oviducts, the uterus, the vagina, the spermatheca, and the fat tissue surrounding the spermatheca (Figure 6.4). The function of these organs is to allow for production, fertilization and deposition of the eggs (Miller, 1950). In *dsx* mutants, these internal reproductive organs and external genitalia are intersexual, demonstrating that *dsx* plays a role in the development of these important sexual characteristics (Hildreth, 1965; Baker and Ridge, 1980).

### Fat Body and Gut

Strong *lacZ* expression was also detected in both the fat body and the gut. In the hindgut, both the male- and the female-specific *dsx* mRNAs were found to be

enriched in comparison to whole fly mRNA, although no enrichment for either mRNA was found in the midgut (FlyAtlas, Chintapalli *et al.*, 2007). Unfortunately, fairly strong expression was detected in the gut of the UAS-*lacZ* line alone, thus it is possible that the observed expression in the gut is not directed by *dsx(15)*-GAL4, but is a consequence of ‘position effects’ on the UAS-*lacZ* line.

The expression in the fat body, on the other hand, is not present in the UAS-*lacZ* X-Gal control staining. *dsx* is known to be expressed in the fat body, where it activates (in females) or represses (in males) the transcription of yolk protein genes *yp-1* and *yp-2* (Burtis *et al.*, 1991; Coschigano and Wensink, 1993; An and Wensink, 1995). This finding is supported by the enrichment of only the female-specific *dsx* mRNA in the adult head (but not the brain) and the larval fat body, as compared to whole fly mRNA (FlyAtlas, Chintapalli *et al.*, 2007). Thus *dsx* likely has an important role in the sexual differentiation of the fat body, which recent studies have shown is critical to the performance of male courtship behaviour (Dauwalder *et al.*, 2002; Fujii and Amrein, 2002; Lazareva *et al.*, 2007).

## CNS

Strong *lacZ* expression was detected in the CNS, and will be discussed in further detail in 6.4.2.

### 6.4.1.2 Embryonic Expression

Previous studies did not examine the expression of *Dsx* in embryos (Lee *et al.*, 2002), although *dsx* expression in the embryo has been detected in the fat body and gonad primordium, and the germ cells and gonads, in Stage 11-12 and Stage 13-16 embryos, respectively, by *in situ* hybridization (BDGP Gene Expression Report; P.Tomancak *et al.*, <http://genomebiology.com/2002/3/12/research/0088.1>). In this chapter, GFP expression was detected in the embryo, however, the embryos examined were not staged. However, these findings, together with the *in situ* data on *dsx* expression in the embryo, suggests that *dsx* may play a role earlier in development than previously thought to specify sexual differentiation.

### 6.4.1.3 Larval Expression

Expression in third instar larvae was strong and widespread, and, no sex-specific expression was detected. The fat body, the CNS (see 6.4.2), and the wing, leg, haltere, eye and antennal imaginal discs (Figure 6.5 and Figure 6.6) all showed expression. The developing testis in male larvae showed no *lacZ* expression; however, expression was seen in the fat body surrounding the testis. This lack of expression in the developing testis is congruent with the lack of expression in the testis of the adult male. The developing ovaries in females were not isolated as a result of their small size; however, given the lack of expression in the ovaries in adults, it seems unlikely that *lacZ* is expressed in the developing ovaries.

## 6.4.2 CNS Expression Generated by *dsx(15)*-GAL4

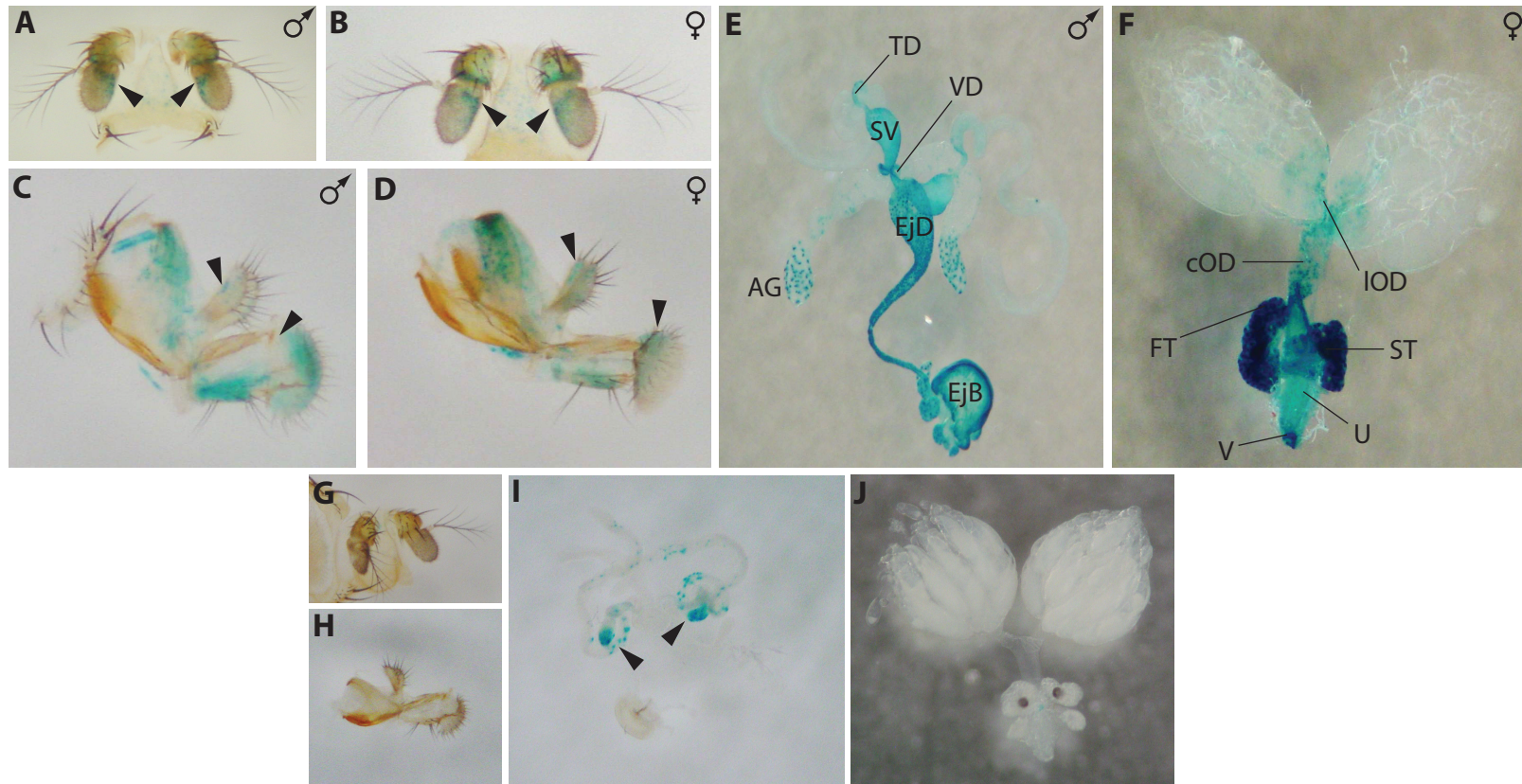
The primary aim of this chapter was to construct a genetic tool to examine the anatomy, development and function of a sexually dimorphic population of *dsx*-expressing neurons in the *Msg*. Thus, the expression driven by *dsx(15)*-GAL4 in the CNS was documented in detail.

### 6.4.2.1 Expression During Development

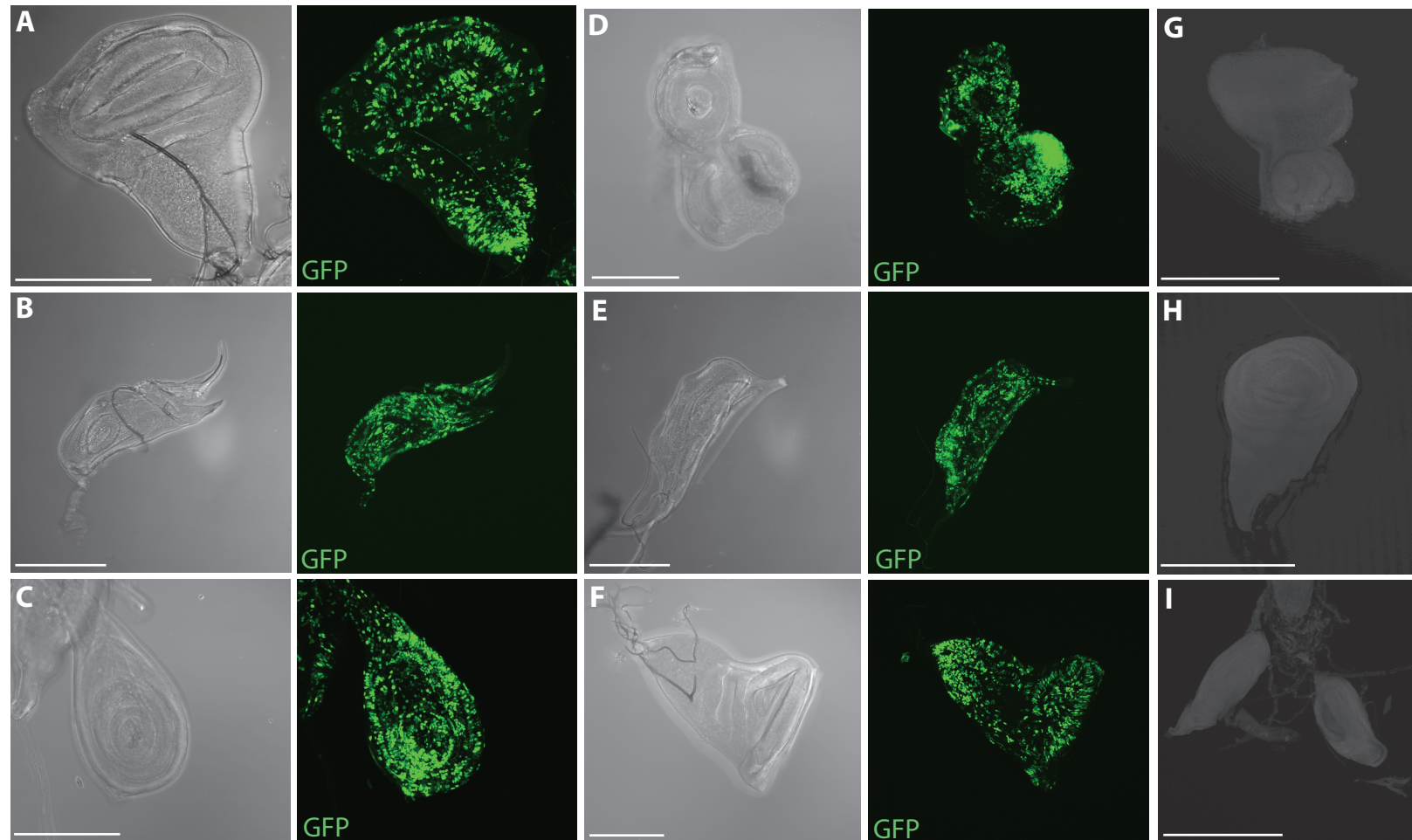
Figure 6.6 shows the expression of a GFP reporter in the CNS driven by *dsx(15)*-GAL4.

#### Larval Expression

No GFP expression was observed in the CNS of either first or second instar larvae (Figure 6.6). These findings are consistent with a previous study of *Dsx* expression, where no expression was observed in either first or second instar larval CNSs (Lee *et al.*, 2002). In third instar larvae expression was detected in the lateral portion of each brain hemisphere, in both males and females. This expression is not consistent with previous reports of *Dsx* expression in third instar larvae, which find that *Dsx* is only expressed in a small number of neurons in the dorsal brain and ventral nerve cord (Lee *et al.*, 2002).

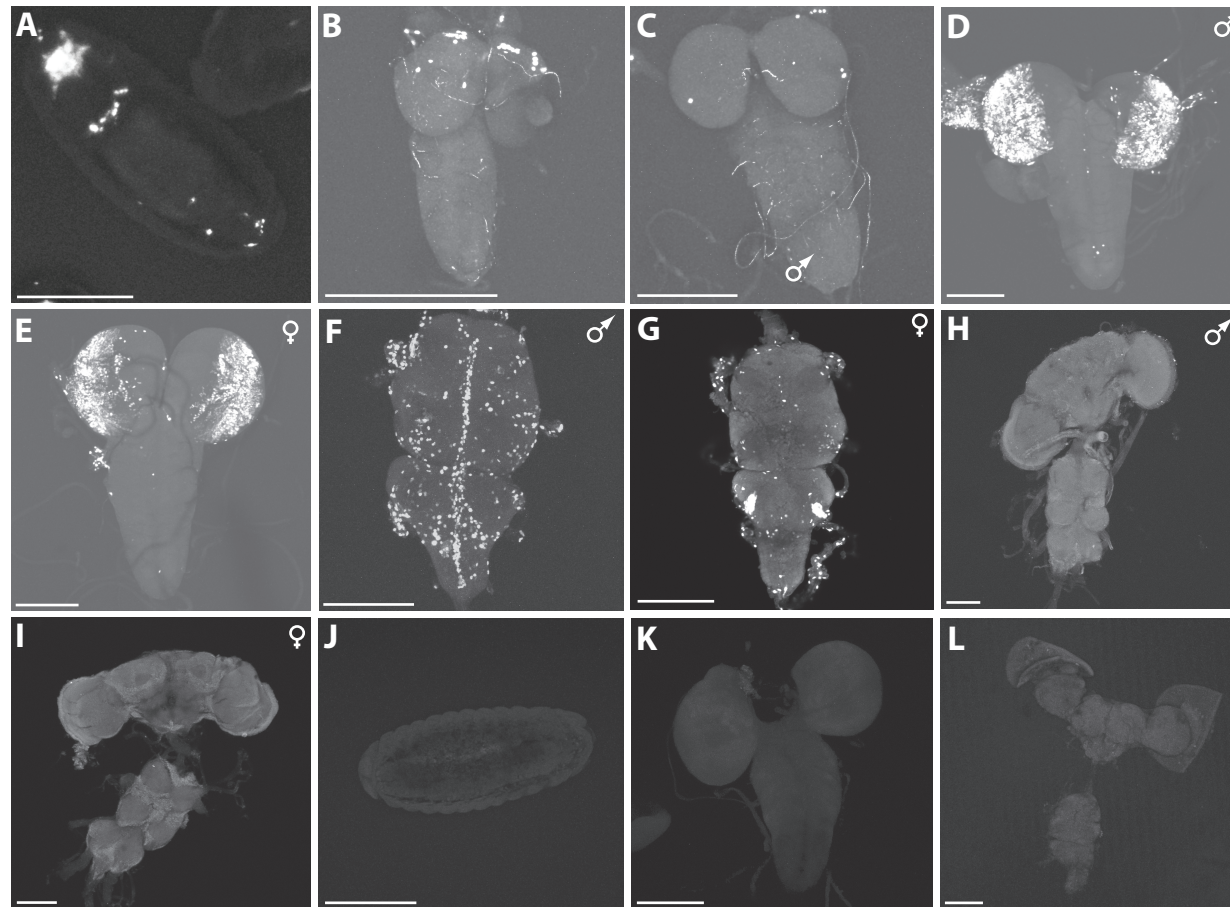


**Figure 6.4** *lacZ* reporter expression driven by *dsx(15)*-GAL4. Expression is indicated by arrowheads. (A,B) *lacZ* expression was detected in the second and third antennal segments, but no differences in expression were apparent between males (A) and females (B). (C,D) *lacZ* expression was detected in the proboscis in the maxillary and labial palps in males (C) and females (D); again, expression was not sexually dimorphic. (E,F) *lacZ* expression was detected in both the male (E) and female (F) internal reproductive organs. Expression in the male reproductive organs was observed in the the seminal vesicles (SV), the testicular duct (TD) the ejaculatory duct (EjD), the vas deferens (VD), the apical tip of the accessory glands (AG) and the ejaculatory bulb (EjB). In females, expression was observed in the lateral oviduct (IOD), the common oviduct (cOD), uterus (U), vagina (V), fat tissue (FT) and the spermatheca (ST). (G-J) Expression generated by UAS-*lacZ* line alone. No expression is seen in the antennae (G), maxillary or labial palps (H) or the ovaries (J). A small amount of expression is seen in the testis (I; arrowheads).



**Figure 6.5-** GFP expression in the larval imaginal discs driven by *dsx(15)*-GAL4. (A-I) Maximum projections of confocal images are shown. Images show phase contrast images of the imaginal discs alongside the GFP expression in the wing disc (A), leg discs (B,C,E), the eye and antennal disc (D), and the haltere disc (F) in third instar male larvae. The expression was not obviously different in female larvae. (G-I) Imaginal discs from UAS-pStinger negative control animals, which show no GFP expression. Scale bar = 200  $\mu$ m.





**Figure 6.6**-GFP expression in the CNS driven by *dsx(15)*-GAL4. (A-L) Maximum Z-projections of confocal images are shown. Larval CNSs and pupal brains are shown with dorsal side up with anterior at the top. Pupal VNCs are shown with ventral side up with anterior at the top. Adult CNSs are shown with the ventral side up and anterior at the top. (A) Embryonic expression. (B) 1st larval instar CNS. (C) 2nd larval instar CNS. 3rd larval instar CNS of a male (D) and female (E). VNCs of a 2-day-old pupal male (F) and female (G). 5-day-old adult CNS of a male (H) and female (I). (J-L) UAS-GFP alone, negative control for embryo (J), 3rd larval instar CNS (K), and 2-day-old pupal CNS (L). Scale bars = 100  $\mu$ m.

## Pupal Expression

The strongest GFP expression was observed in the CNS of 2-day-old pupae, and was observed in the brain and the ventral thoracic ganglia of the CNS (Figure 6.6 and Figure 6.7). In the CNS, the GFP-expressing cells appeared to fall into two classes based on their position within the brain. The first class of cells was located primarily on the periphery of the brain, where individual cells were fairly large in size, and their distribution over the surface of the brain was fairly uniform. In the ventral thoracic ganglia, only this first class of cells was found. The second type of GFP-expressing cell, found only in the posterior brain, was smaller, and was located in four distinct clusters in the posterior brain (Figure 6.7).

## Adult Expression

No GFP expression was detected in the CNS of adult males or females (Figure 6.6). Previous reports have found both *dsx* transcripts and protein to be expressed in adult males and females at extremely low levels (Wolfner and Baker, 1988; Lee *et al.*, 2002). The result in this study supports these previous findings, suggesting that *dsx* transcription peaks during the pupal period, and is dramatically reduced in adults.

### 6.4.2.2 Sex-Specific Expression

Lee *et al.* (2002) found that the number of Dsx-expressing neurons in the CNS was sexually dimorphic in the posterior brain, the Msg and the Abg. Given that *dsx(15)*-GAL4 directs GFP expression in identifiable clusters in the posterior brain, these clusters were examined more closely, to determine if the number of GFP-expressing cells was sexually dimorphic. Figure 6.7 shows the GFP-expressing neurons in the posterior brain in both males and females, where a clear difference can be seen in each of four distinct clusters (two per hemisegment). In males, the number of GFP-expressing cells in males in Cluster 1 (C1, closer to the midline) was  $29.2 \pm 4.7$ , significantly greater than the  $4.0 \pm 0.8$  C1 cells counted in females ( $n=4$ ;  $p<0.05$ ). In Cluster 2 (C2, more laterally placed), females also had fewer cells, with only  $10.5 \pm 2.1$ , compared to  $52.2 \pm 3.2$  in males ( $n=4$ ;  $p<0.05$ ). Thus females have significantly fewer cells in this

region of the posterior brain than males. What type of cells do these sexually dimorphic clusters contain?

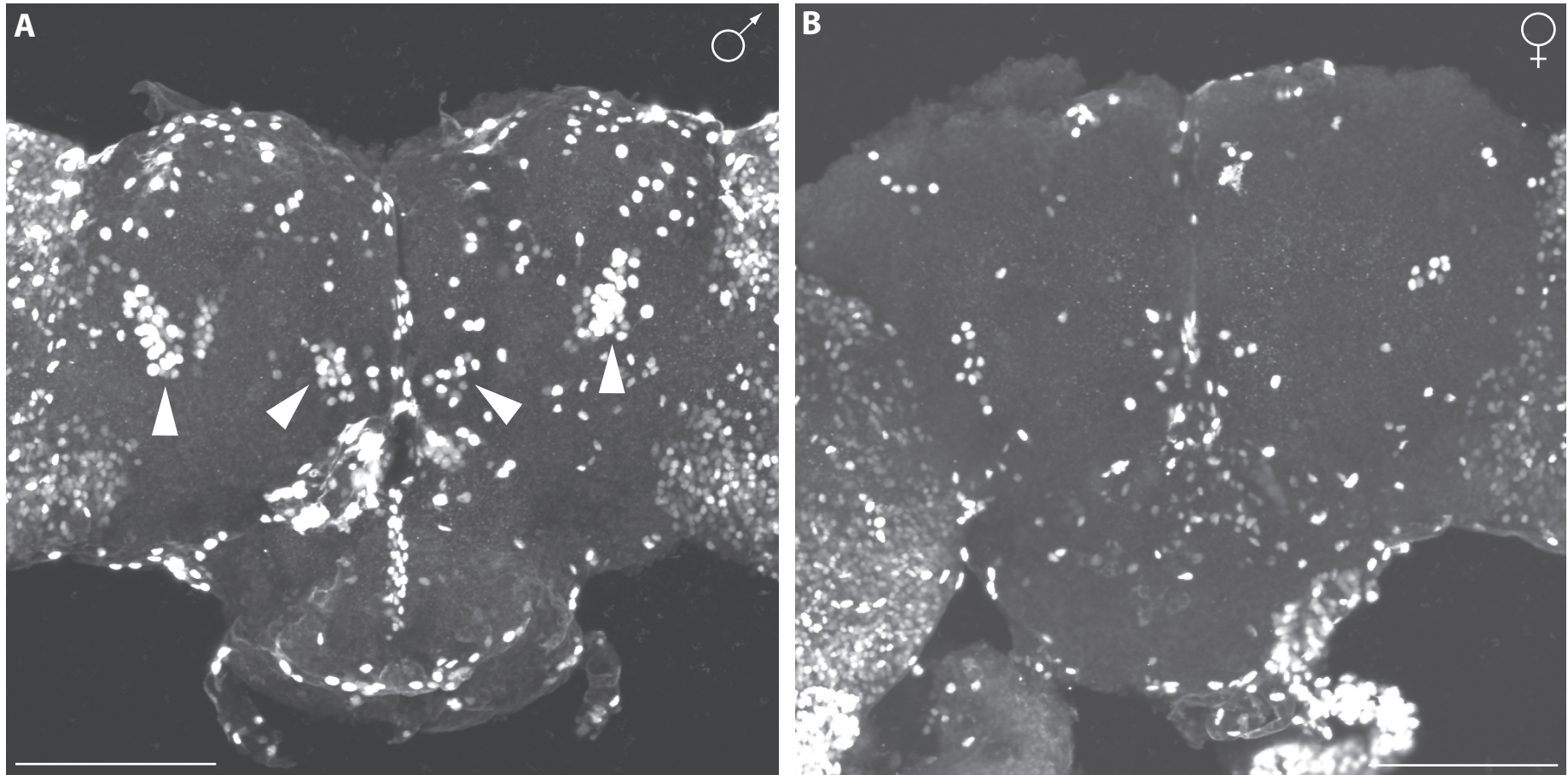
#### 6.4.2.3 What is the Identity of the Sexually Dimorphic GFP-Positive Cells?

Previous reports showed that *Dsx* is expressed both in glia and in neurons (Lee *et al.*, 2002). Given that two classes of GFP-expressing cells were found, it seems possible that *dsx(15)*-GAL4 directs expression in both glia and neurons. To test this hypothesis, the posterior brain was co-labelled with anti-GFP and anti-Repo (a glial cell marker; Xiong *et al.*, 1994; Halter *et al.*, 1995) (Figure 6.8). Some of the cells in the first class of GFP-expressing cells co-localize with Repo, suggesting that these larger, more dispersed cells are indeed glia. None of the smaller, clustered GFP-expressing cells showed co-localization with Repo, suggesting that these cells are neurons. In the future, to confirm that these smaller, clustered cells are neurons, the expression of GFP will be co-localized with Elav, a marker for postmitotic neurons (Yao *et al.*, 1993). Thus *dsx(15)*-GAL4 directs expression in both neurons and glia in 2-day-old pupae.

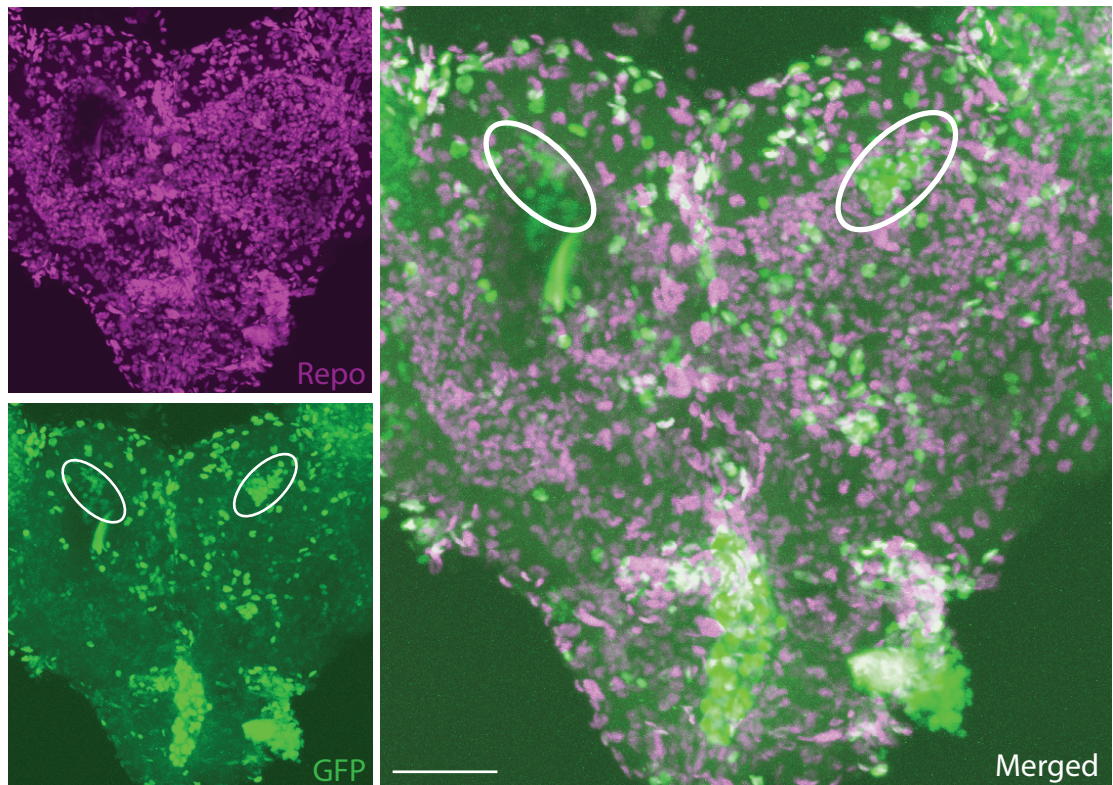
#### 6.4.2.4 Is the Expression Driven by *dsx(15)*-GAL4 Congruent with *Dsx* Expression?

As described in the previous section, the small, clustered GFP-expressing cells found only in the posterior brain are likely to be neurons; whereas many of the larger, more dispersed GFP-expressing cells, found throughout the brain and thoracic ganglia are likely glia. Given that the aim of constructing the promoter-GAL4 fusion transgene was to examine the anatomy, development and function of *dsx*-expressing neurons, and that the posterior brain is the only region in which *dsx(15)*-GAL4 directs expression in cells that appear to be neurons, it was determined whether the GFP expression in the posterior brain was congruent with *Dsx*. Figure 6.9 shows the co-localization of GFP and *Dsx* in the posterior brain of 2-day-old male pupae. In females, no *Dsx*-expressing cells in the posterior brain were located, and thus co-localization was not attempted. Given that these *Dsx*-expressing cells in the posterior brain were shown by Lee *et al.* (2002) to be neurons, these results demonstrate that *dsx(15)*-GAL4 directs expression of a reporter in *dsx*-expressing neurons in the posterior brain, and that it is an excellent tool with which to study the development and function of these neurons.



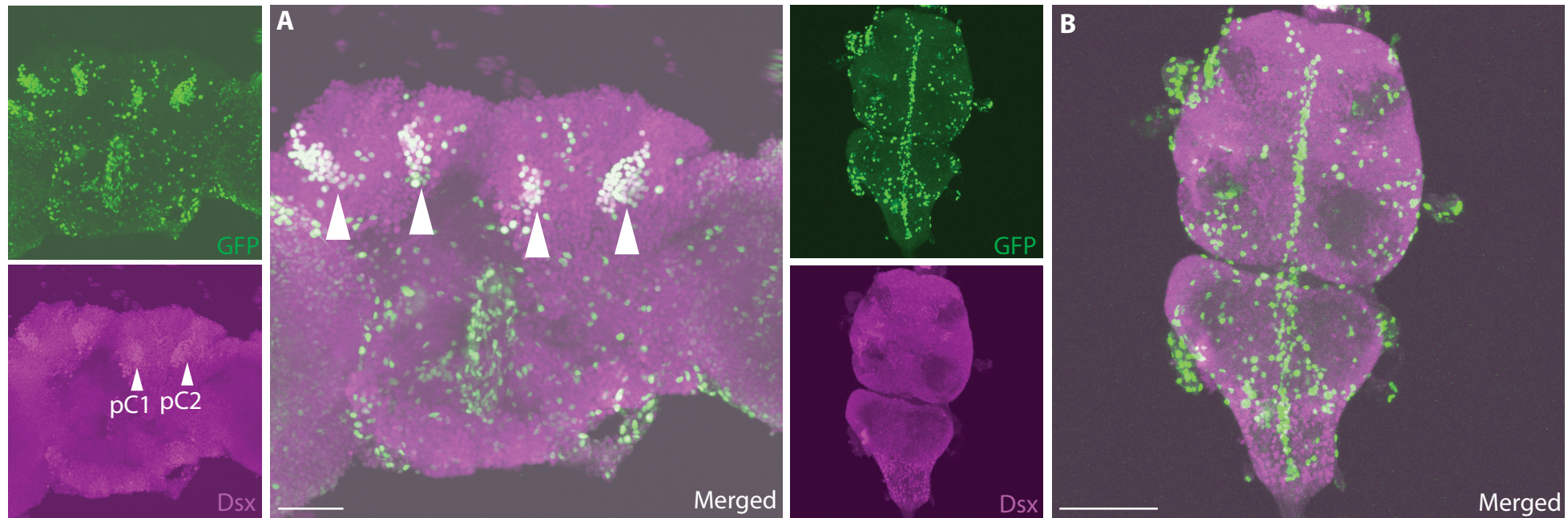


**Figure 6.7**-Sex-Specific Expression of GFP in the Posterior Brain. Maximum Z-projections of confocal images of GFP expression driven by *dsx(15)*-GAL4 in the posterior brain in a 2-day-old pupal male (A), and female (B), are shown. Anterior is at the top of the image, with dorsal side up. Arrowheads indicate sex-specific neuronal clusters in the male pupal CNS. Scale bars = 100  $\mu$ m.



**Figure 6.8-** Co-localization of GFP with Glial Cell Marker Repo in the Posterior Brain. Maximum Z-projections of confocal images are shown. 2-day-old male pupal brain stained with anti-GFP and anti-Repo. Co-localization is present in a number of GFP-positive cells. The white circle indicates the cluster of GFP-positive cells that does not co-localize with Repo. Scale bar = 50  $\mu\text{m}$ .





**Figure 6.9**-Co-expression of GFP with Dsx in the Posterior Brain. Maximum Z-projections of confocal images are shown. (A) Co-localization of Dsx and GFP is seen in four distinct clusters in the posterior brain. Near perfect congruence between GFP and Dsx is seen in both the pC1 and pC2 clusters of *dsx*-expressing neurons (nomenclature as per Lee *et al.*, 2002). Arrowheads indicate regions where co-localization is seen. Sample is oriented with dorsal side up with anterior to the top. (B) Co-expression between Dsx and the GFP reporter was not observed in the VNC. Sample is oriented with ventral side up with anterior at the top. Scale bar = 50 μm.

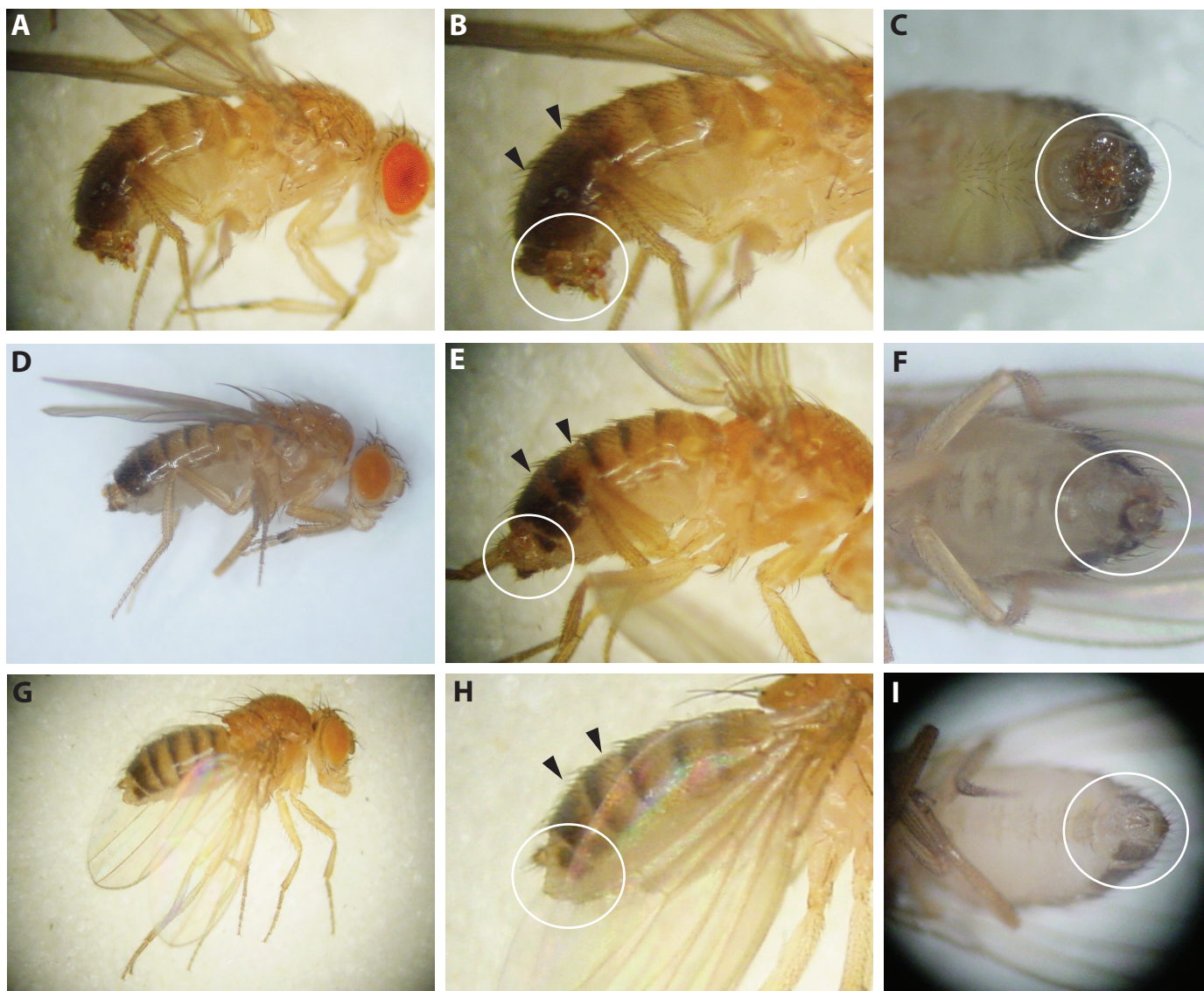
## 6.5 Feminization of Male Flies with *dsx(15)*-GAL4

The previous sections described the spatial and temporal expression directed by the *dsx(15)*-GAL4 transgene. Fairly widespread expression was seen in the body, with a number of structures involved in sex-specific behaviours showing reporter expression. In addition, reporter expression was also detected in the CNS, where the expression of the GFP reporter in clusters of neurons in the posterior brain was congruent with Dsx expression in that region. Therefore, to determine whether the expression dictated by *dsx(15)*-GAL4 was physiologically relevant, the consequences of feminizing these regions using the UAS- *tra* transgene were examined. Normally, males do not express Tra protein, and as a result they express Fru<sup>M</sup> and Dsx<sup>M</sup>, the male-specific isoforms of *fru* and *dsx*, respectively (Nagoshi *et al.*, 1988; Hoshijima *et al.*, 1991; Ryner *et al.*, 1996; Lee *et al.*, 2002). However, when UAS- *tra* expression is driven by GAL4 in males, any cells in which GAL4 driver is expressed are ‘feminized’, that is, those cells express the female isoform of *dsx*, Dsx<sup>F</sup>, and no Fru<sup>M</sup> (Ferveur *et al.*, 1995; Ferveur and Greenspan, 1998). Indeed, when *tra* is ectopically expressed in males, chromosomal males are transformed into phenotypic females (McKeown *et al.*, 1988).

### 6.5.1 External Feminization

Sex determination in *Drosophila melanogaster* is thought to be cell autonomous (reviewed by Oliver, 2002; Billeter *et al.*, 2006a), with the exception of the germline, which suggests that the expression of *tra* and *dsx* is required in most cells to determine sex. Although the spatial and temporal expression pattern of *tra* remains unknown, in the CNS, only a restricted number of neurons express *dsx*, suggesting that *dsx* expression is not required in all neurons to determine sex. To determine if the same is true in tissues outwith the CNS, *dsx(15)*-GAL4 was used to determine if its widespread (but not ubiquitous) expression could direct the transformation of a chromosomal male into a ‘pseudofemale’. If so, it would suggest that sex determination is not required in all cells, rather it is only necessary in subsets of cells which will ultimately perform sex-specific functions. Moreover, the ability of *dsx(15)*-GAL4 to transform chromosomal males into ‘pseudofemales’ would suggest that it accurately reflects the expression of Dsx.





**Figure 6.10**-Feminization of Males with UAS-*tra*. (A-C) Wild-type 5-day-old CS male. (A) Lateral view of male fly is shown. (B) Closer view of male abdomen. Male-specific pigment in A5 and A6 is indicated by arrowheads. Circle highlights the side view of male genitalia. (C) Ventral view of male genitalia, indicated by white circle. (D-F) 5-day-old feminized male. (D) Lateral view of feminized individual. (E) Feminized pigment in A5 and A6 segments is indicated by arrowheads, and the abnormal genitalia are indicated by the white circle. (F) Ventral view of abnormal genitalia, indicated by white circle. (G-I) 5-day-old wild-type CS female. (G) Lateral view of UAS-*tra* control female. (H) Female-specific pigment in A5 and A6 is indicated by arrowheads, and lateral view of female genitalia is indicated by the white circle. (I) Ventral view of female genitalia, indicated by white circle.

Figure 6.10 shows a wild-type female and male, alongside a chromosomally male fly partially transformed into a female. Although the genitals of the transformed males loosely resembled those of wild-type males, they were rotated approximately 180°. These transformed males still possessed sex combs; however, the most obvious transformation was the difference in the abdominal pigmentation of these males, which was very similar to the pigmentation seen in wild-type females (Figure 6.10). No such abnormalities or transformations were observed in either UAS-*tra*/+ males, or in *dsx(15)*-GAL4/UAS-*tra* females.

An examination of the internal reproductive organs of these transformed males revealed that they possessed essentially wild-type male internal organs, with the exception of the testes, which were occasionally absent or malformed. It could not be determined whether these abnormal structures were functional, as these transformed males were sterile (n=21), likely as a result of the rotated genitalia.

### 6.5.2 Behavioural Effects of Feminization

Males carrying the *dsx(15)*-GAL4 transgene, as well as the UAS-*tra* transgenic flies are all in a *w*<sup>1118</sup> background, as this allowed for easy selection of transformants carrying the *mw*<sup>+</sup> transgene. However, flies lacking wild-type pigmentation in their eyes (as in *w* mutants) have been shown to be deficient in courtship (Sturtevant, 1915). Therefore, prior to examining the behaviour of these flies, it was necessary to put the *dsx(15)*-GAL4 into a wild-type background to eliminate the possibility of strong effects of genetic background on courtship behaviour; and to introduce a wild-type copy of the *w* gene onto the *dsx(15)*-GAL4-containing X chromosome, to ensure wild-type eye pigmentation in the experimental males.

Females containing the *dsx(15)*-GAL4 transgene were crossed to a strain of *w*<sup>1118</sup> flies previously isogenized to a CS wild-type background. Female progeny from this cross containing the *dsx(15)*-GAL4 transgene were then crossed again to 'Cantonized' *w*<sup>1118</sup> males. This cross was then repeated for six generations, to ensure that the *dsx(15)*-GAL4 line was fully isogenized to a wild-type genetic background. Then, in order to introduce a functional copy of *w* into the X chromosome on which *dsx(15)*-GAL4 is located, a female containing the *dsx(15)*-GAL4 transgene was crossed to a wild-type CS male. Female progeny from this

cross were taken and crossed to males with genotype *FM7;+;+*, a first chromosome balancer line (which was made isogenic to CS). Male progeny from this cross with wild-type eye pigmentation, without *Bar* eyes were taken and crossed singly to *FM7;+;+* females. The male and female progeny from this cross with *Bar* eyes were crossed, and stocks for each single male were set up. In order to determine which stocks contained the *dsx(15)*-GAL4 transgene, each individual line was crossed to UAS-*lacZ*, and X-Gal staining revealed the desired stocks.

Unfortunately, the time taken for these crosses precluded any behavioural analysis prior to the end of the allotted time in the laboratory. However, upon returning to the lab, these experiments will be performed immediately.

## 6.6 Discussion

This chapter described the construction and characterization of a promoter-GAL4 fusion transgene capable of directing expression in *dsx* neurons, with the aim of investigating the anatomy, development and function of a population of sex-specific neurons in the mesothoracic ganglion.

### 6.6.1 Constructs and Transformants

Generating transgenic flies with very large *P*-elements is difficult, as *P*-element transformation efficiency drops significantly when larger constructs are used. The expected germ-line transformation frequencies for constructs of less than 15 kb are between 5-15% of the fertile injected embryos (Spradling, 1986). When choosing the fragment of the *dsx* promoter to insert into the *P*-element transformation vector, size was a limiting factor, as the entire set of enhancers was potentially spread out over a large genomic region (Blackman *et al.*, 1991; Pirodda, 1995; reviewed by Sipos and Gyurkovics, 2005). Therefore, a fragment approximately 15 kb in size lying upstream of the putative *dsx* transcriptional start site was inserted into a modified version of the insulated *P*-element transformation vector pStinger (Barolo *et al.*, 2000; Billeter and Goodwin, 2004).

The final *dsx*-GAL4 construct was just below 30 kb in size, where approximately 300 embryos were injected and fourteen independent transformation lines were

obtained. Barolo *et al.* (2000) reported a lower transformation frequency for the insulated pStinger vectors, presumably as a result of the enhancer blocking function of the insulator sequences disrupting the transcription of the gene into which the *P*-element was inserted. Provided this were true, it may have been possible to obtain more than fourteen independent transformant lines, however, the number of transformants was more than sufficient to proceed with further characterization of the lines.

### **6.6.2 Non-Neuronal Expression Dictated by *dsx(15)*-GAL4**

When the *dsx(15)*-GAL4 lines were crossed to transgenic UAS-*lacZ* flies, only one line was found to drive the expression of *lacZ*. This is surprising, as one of the major advantages of using insulated vectors is to decrease line-to-line variation in the expression patterns that result from ‘position effects’ on the transgenes. It therefore seems possible that many of the *dsx* promoter-GAL4 fusions were either inserted into relatively inactive genomic locations, preventing the expression of GAL4, or that there are sequences within the *dsx* promoter sequence chosen that are actively repressed in the absence of enhancers elsewhere in the *dsx* locus. Alternatively, it is also possible that thirteen of the independent insertion lines underwent some sort of internal re-arrangement or deletion, and were not inserted intact into the chromosome (Dominguez and Albornoz, 1996; Albornoz and Dominguez, 1999). To investigate this possibility, a Southern blot would need to be performed on all fourteen independent insertions of *dsx(15)*-GAL4, to ensure that the entire *dsx* promoter-GAL4 fusion was intact in these lines.

When a reporter was used to characterize the expression dictated by the promoter element contained in *dsx(15)*-GAL4, the reporter was found to be expressed in several locations. The *lacZ* reporter was found in sensory structures such as the antennae and maxillary palps, in both male and female internal reproductive structures, the fat body and the CNS. All of these structures play roles in the determination or modulation of sex-specific courtship and/or reproductive behaviours, thus finding that a *dsx* promoter dictates expression in these regions is perhaps not surprising. The full pattern of Dsx expression in non-neuronal tissues has not been documented, thus the expression driven by the *dsx(15)*-GAL4 promoter may give clues as to the location of Dsx expression.



Ideally, the expression of Dsx in tissues outwith the CNS would have been documented in this chapter, to determine whether the expression dictated by *dsx(15)*-GAL4 does indeed reflect Dsx expression. However, the quantities of anti-Dsx antibody in this study were extremely limiting, and the congruence of the reporter with Dsx was only documented in the CNS. More antibody has now been made, and will be used in the future to produce not only a full spatial and temporal description of Dsx expression, but also to determine where and when *dsx(15)*-GAL4 re-capitulates the expression of Dsx.

### **6.6.3 Expression Dictated by *dsx(15)*-GAL4 in the CNS**

*dsx(15)*-GAL4 was found to drive expression in both neurons and glia, which is consistent with a previous report suggesting Dsx is expressed in both cell types in the CNS (Lee *et al.*, 2002). Reporter expression was observed in the CNS in embryos, third instar larvae and in 2-day-old pupae, results consistent with previously reported temporal patterns of Dsx expression (Lee *et al.*, 2002). However, the only stage at which the expression of Dsx and the GFP reporter were found to be congruent was 2-day-old pupae, in cells located in the posterior brain. These cells were shown to be neurons by Lee *et al.* (2002), a finding confirmed by results in this chapter demonstrating that these cells do not express a glial-cell marker Repo (Xiong *et al.*, 1994; Halter *et al.*, 1995). Thus *dsx(15)*-GAL4 drives expression in Dsx neurons located in the posterior brain.

The posterior region of the brain has been implicated in the initiation of male courtship behaviour, and in licking, tapping and wing extension (Hall, 1977, Hall, 1979; Ferveur and Greenspan, 1998). In this study it was shown that Dsx and Fru<sup>M</sup>, two sex determination genes are co-expressed in three regions of the CNS, including the posterior brain. As shown in this study and by Billeter *et al.* (2006b), sexually dimorphic populations of neurons are present in the other regions in which Dsx and Fru<sup>M</sup> are co-expressed, where both Dsx and Fru<sup>M</sup> are required to create these differences. These results suggest that sexually dimorphic neuronal populations are created in regions of Dsx and Fru<sup>M</sup> co-expression. Indeed, in the chapter it was shown that a sex-specific population of neurons is also present in the posterior brain. Although the factors influencing the development of this neuronal population were not determined, it seems likely that as with the *Msg* and *Abg*, both Dsx and Fru<sup>M</sup> will be required. Provided

this were true, *dsx(15)*-GAL4 is an excellent tool with which to examine the anatomy, development and function of the sexually dimorphic population of neurons in the posterior brain.

For example, single cell mosaic analysis would allow the projections of individual neurons in this sex-specific population of neurons to be examined, where these projections may give insight as to their function. In addition, given its location on the X chromosome, *dsx(15)*-GAL4 can be put into a *dsx*-, *fru*- or cell death-mutant genetic background, allowing the investigation of the mechanism by which the sexually dimorphic population of neurons is created. This experiment is critical, as prior attempts to make a recombinant third chromosome bearing either a *dsx*- or *fru*-mutation in combination with the *rpr* cell death-mutation failed. Finally, single-cell mosaics can be created with *dsx(15)*-GAL4 using the mosaic analysis with a repressible cell marker (MARCM) method, which creates single-cell clones by mitotic recombination that are both mutant for a desired gene, and marked by GFP (Lee and Luo, 1999). Previously, Kimura *et al.* (2005) used this technique to show that the morphology of single neurons in the brain was altered by making these single neurons mutant for Fru<sup>M</sup>. Thus in a similar manner, single neurons in the posterior brain can be mutant for either *tra*, *fru* or *dsx*, and marked with GFP, to investigate the effects of each gene on the neurobiological properties and development of these neurons. In addition, the courtship behaviour of these mosaic flies can be observed and analyzed prior to dissection, to correlate aberrant behaviours with alterations in neuronal morphology and function. In this way, a better understanding of the how genes influence the development of sexually dimorphic neuronal structures and function will be gained, along with a better understanding of how these neuronal properties give rise to sex-specific behaviours.

#### **6.6.4 *dsx(15)*-GAL4 and the Mesothoracic Ganglion**

Although *dsx(15)*-GAL4 was found to direct the expression of a reporter in clusters of Dsx-expressing neurons in the posterior brain, no expression in neurons was observed in the Msg. This is unfortunate, as the primary aim of creating the *dsx(15)*-GAL4 transgene was to investigate the neurobiological properties of a sexually dimorphic population of neurons in the Msg implicated in the performance of courtship song, as described in the previous chapter. This

may be a result of the *dsx* promoter fragment chosen, where the 15 kb fragment chosen may not contain enhancers capable of directing expression in the *Msg*. Given that only 15 kb was cloned into the *P*-element transformation vector, the relevant sequences must be found elsewhere. In order to investigate these sexually dimorphic neurons in the *Msg*, another genetic tool containing the relevant enhancers must be constructed, using one of two strategies: 1) making further promoter-GAL4 fusion transgenes, or 2) inserting GAL4 into the *dsx* locus by homologous recombination. If the former option is chosen, multiple large genomic fragments would need to be inserted into the *P*-element for germline transformation, and the screening of insertion lines and characterization would proceed again as described in this chapter. Alternatively, by inserting GAL4 directly into the *dsx* locus by homologous recombination, the expression of GAL4 would be dictated by the complete set of enhancers surrounding the *dsx* locus, and the expression of GAL4 would reflect the expression of *Dsx* (as per Stockinger *et al.*, 2005). Therefore, in the next chapter, the insertion of GAL4 into the *dsx* locus will be described.

## 6.7 Conclusions

The results presented in this chapter show that the *dsx* promoter-GAL4 fusion transgene is capable of driving the expression of a reporter in a number of tissues during development. Many of these tissues are either sexually dimorphic (*eg.* internal reproductive organs), or have been implicated in sex-specific roles (*eg.* antennae and maxillary palps). In the CNS, the co-expression of *Dsx* and the GFP reporter was examined, and it was determined that reporter expression was congruent with *Dsx* expression in four clusters of *Dsx*-expressing neurons in the posterior brain. Furthermore, it was determined that the number of neurons in these four clusters in the posterior brain was sexually dimorphic, with females having far fewer neurons than males. Thus the posterior brain, like the *Msg*, has a sexually dimorphic neuronal population in a region of *Dsx* and *Fru<sup>M</sup>* co-expression. Given these similarities, it can be suggested that the mechanism by which the sex-specific population of neurons is specified in the posterior brain may be similar to the creation of the sexually dimorphic population of neurons in the *Msg*. If so, *dsx(15)*-GAL4 can be used to examine the sexually dimorphic neurobiology, development, and function of the neurons in the posterior brain,

and these findings may apply more generally to other sexually dimorphic populations in the CNS that are dependent on both Fru<sup>M</sup> and Dsx.

Although the expression dictated by *dsx(15)*-GAL4 is congruent with Dsx in the posterior brain, no reporter expression was observed in the *Msg*. Therefore, another tool is required to investigate how the sexually dimorphic population of Dsx-expressing neurons in the *Msg* controls song production.

## **7 Generation of $dsx^{GAL4}$ by Homologous Recombination**

## 7.1 Introduction

In the previous chapter, the generation of a *dsx* promoter-GAL4 fusion transgene was described. The aim of making this construct was to generate a genetic tool capable of driving expression in a sex-specific cluster of *dsx* neurons, so that the anatomy, development and function of these dimorphic neurons could be investigated. *dsx(15)*-GAL4 was found to dictate the expression of a reporter in two clusters of *dsx*-expressing neurons, called pC1 and pC2 (nomenclature as per Lee *et al.*, 2002), in the posterior brain. However, the primary aim of this thesis is to investigate the neurobiology and genetics underlying the sex-specific production of courtship song, and *dsx(15)*-GAL4 does not drive expression in a sex-specific cluster of neurons in the *Msg*, a region of the CNS strongly implicated in song production (von Schilcher and Hall, 1979). Therefore, although *dsx(15)*-GAL4 can be used in future studies to examine the development and function of the sex-specific neuronal clusters in the posterior brain (see 8.1.3 and 8.2.1), another approach must be taken to generate a GAL4 driver capable of directing expression in the sex-specific cluster of *dsx*-expressing neurons in the *Msg*.

The major problem with generating a *dsx* promoter-GAL4 fusion construct was the relatively large size of the *dsx* locus (~40 kb). The 15 kb fragment of DNA inserted upstream of GAL4 did not contain the necessary enhancers for *dsx*'s *Msg* expression, and the generation of additional *dsx* promoter-GAL4 fusions may be equally unsuccessful, as it is not clear where these critical enhancers are located. Recently, the technique of gene targeting by homologous recombination has been described in *Drosophila melanogaster*, a technique which enables the targeted insertion of GAL4 directly into a desired locus (reviewed by Bi and Rong, 2003). Therefore, with the aim of generating a line capable of driving expression in all *dsx* neurons, specifically the dimorphic population of neurons in the *Msg*, gene targeting by homologous recombination was used in this study to insert GAL4 into the *dsx* locus.

### 7.1.1 Targeting GAL4 to the *dsx* Locus

Stockinger *et al.* (2005) and Manoli *et al.* (2005) used homologous recombination to insert GAL4 into the *fru* locus, and GAL4 was found to direct the expression of

a reporter in all  $Fru^M$  neurons. The sexually dimorphic population of neurons in the *Msg* expresses  $Fru^M$ , and  $fru^{GAL4}$  is able to drive expression in these sex-specific neurons; however,  $fru^{GAL4}$  directs expression in many neurons in the *Msg*, making the isolation, manipulation and description of the 23 sex-specific neurons more difficult. Therefore, following a similar strategy to that described by Stockinger *et al.* (2005), GAL4 will be inserted into the *dsx* locus, generating a GAL4 driver capable of dictating expression in all *dsx* neurons, including the population of sex-specific neurons in the *Msg*.

Gene targeting by homologous recombination can be achieved by following either of two strategies: ends-in or ends-out (see Figure 1.6 and Figure 1.7). Ends-out homologous recombination has been reported to be less efficient in yeast and mammalian cells (Hasty *et al.*, 1991; Hastings *et al.*, 1993), and is generally used to make a null mutation in a gene of interest. In addition, unless recombination sites, such as loxP, are engineered into the donor construct, the marker will remain in the targeted locus (Stockinger *et al.*, 2005; reviewed by Bi and Rong, 2003). Ends-in gene targeting, on the other hand, is typically used to make more subtle mutations in a gene of interest, such as small deletions or amino acid substitutions (reviewed by Bi and Rong, 2003). Moreover, the marker is eliminated from the gene of interest in a second step of the ends-in strategy, ensuring that it does not interfere with the phenotype under investigation.

In this study, the ends-in strategy of homologous recombination was chosen to insert GAL4 into the *dsx* locus, given that it may be the more efficient of the two strategies, that it was capable of making small changes in the *dsx* locus, and that the marker gene was eliminated from the targeted locus. This latter point is important, as the marker present on the *P*-element donor construct used in this study is *mw*, which has been previously shown to trigger high levels of inter-male courtship (Zhang and Odenwald, 1995). Given that the line containing GAL4 at the *dsx* locus will eventually be used in studies of courtship behaviour, the *mw* marker gene must be eliminated to prevent its interference in future studies of courtship behaviour. Also, given that a number of successful studies using gene targeting by ends-in homologous recombination have been published, and that targeting vectors are readily available (Rong and Golic, 2000; Rong and Golic, 2001; Rong *et al.*, 2002; Stockinger *et al.*, 2005; reviewed by Bi and Rong, 2003), GAL4 will be inserted into the *dsx* locus in a similar manner to that described by

Stockinger *et al.* (2005), with the addition of a second step to remove the duplication at the *dsx* locus, and to eliminate the *mw* marker gene.

## 7.2 Generation of Gene-Targeting ‘Donor’ Construct

### 7.2.1 Donor Construct

#### 7.2.1.1 Vector

An unpublished gene targeting vector for ends-in homologous recombination called pED22 was generously provided by B. Dickson (IMBA, Austria) for the generation of a *dsx*<sup>GAL4</sup> (Figure 7.1). The *mw* gene is present as a selectable marker for both steps of the gene targeting strategy. *P*-element transposition sequences are present at the extreme 5' and 3' ends so that it can be inserted at random into the genome prior to the first step of the gene targeting strategy. Just inside each of these transposition sequences at the 5' and 3' ends are the FRT sites to mediate the excision of the construct from the genome in the first step of the gene targeting process. Regions of homology are inserted into each of three multiple cloning sites. An *I-SceI* site-specific endonuclease recognition site is present between the first and second regions of homology, to induce a double-stranded break during the first stage of the targeting process. Finally, an *I-CreI* site-specific endonuclease recognition site is present between the third region of homology and the *mw* marker gene, to create a double-strand break during the second step of the targeting process to resolve the gene duplication.

#### 7.2.1.2 Design

A number of studies in other targeting systems have shown that the donor:target homology can affect the frequency of homologous recombination (Shen and Huang, 1986; Deng and Capecchi, 1992; Dray and Gloor, 1997; Papadopoulou and Dumas, 1997; Gray and Honigberg, 2001). Indeed, regions of homology ranging from 2.5 kb to 8.9 kb have been used in successful gene targeting experiments; however, the homology is generally around 4 kb (Rong and Golic, 2000; Rong and Golic, 2001; reviewed by Bi and Rong, 2003). Therefore, three regions of homology of at least 3.5 kb in size were chosen from the *dsx* genomic locus to be inserted into the targeting vector pED22 (Figure 7.1 and Figure 7.2).



In addition to size, the desired end product influenced the choice of fragments from the targeted locus. In this study, GAL4 was chosen to replace the first coding exon; however, sex-specific splicing events downstream of this exon required the preservation of the splice donor site at the 3' end of this exon. Therefore, two regions of homology were chosen immediately upstream of the translation start site, and the final region included the splicing enhancers at the 3' end of this first coding exon (Figure 7.1).

#### **7.2.1.3 Isolation and Verification of Homologous Fragments**

Each fragment was amplified by PCR using the high-speed and -fidelity Phusion polymerase (New England Biolabs), purified and cloned into the TOPO-TA Cloning vector (Invitrogen), as described in 2.4.11. Plasmids with the desired insert were purified, and sequenced twice to verify that no mutations were induced during PCR amplification.

#### **7.2.1.4 Cloning Strategy**

The cloning of the *dsx*<sup>GAL4</sup> transgene is shown in Figure 7.1. The fragments were inserted sequentially into pED22, and the final product, called pED22-*dsx*, was verified by restriction digest.

### **7.2.2 Transformants Containing the 'Donor' Construct**

Prior to the start of the process of targeting the insertion of GAL4 to the *dsx* locus, the *P*-element donor construct must be inserted by *P*-element transformation into the genome. The final pED22 construct containing three regions of homology to the *dsx* locus was microinjected into embryos (Genetic Services Inc.; Cambridge, MA). Transformants were selected on the basis of eye colour conferred by the *mw* marker on the donor construct. Eleven independent transformant lines were recovered, and the chromosome of insertion for each was determined (Table 7.1). When a locus on the third chromosome is being targeted, *P*-element donor insertion lines on the second or X chromosomes are chosen, to allow the differentiation in later steps between events where the *P*-element has re-inserted at the original locus, and events where the donor has inserted at the desired locus. Thus, a single transformant line on the second

chromosome (#2) was chosen to proceed with gene targeting by homologous recombination.

Line Number	Chromosome	Line Number	Chromosome
1	X	7	2
2	2	8	2
3	3	9	3
4	2	10	2
5	2	11	2
6	X		

**Table 7.1**-Independent *P*-element Transformant Lines for pED22-*dsx*. The line number and chromosome of insertion are shown in the table.

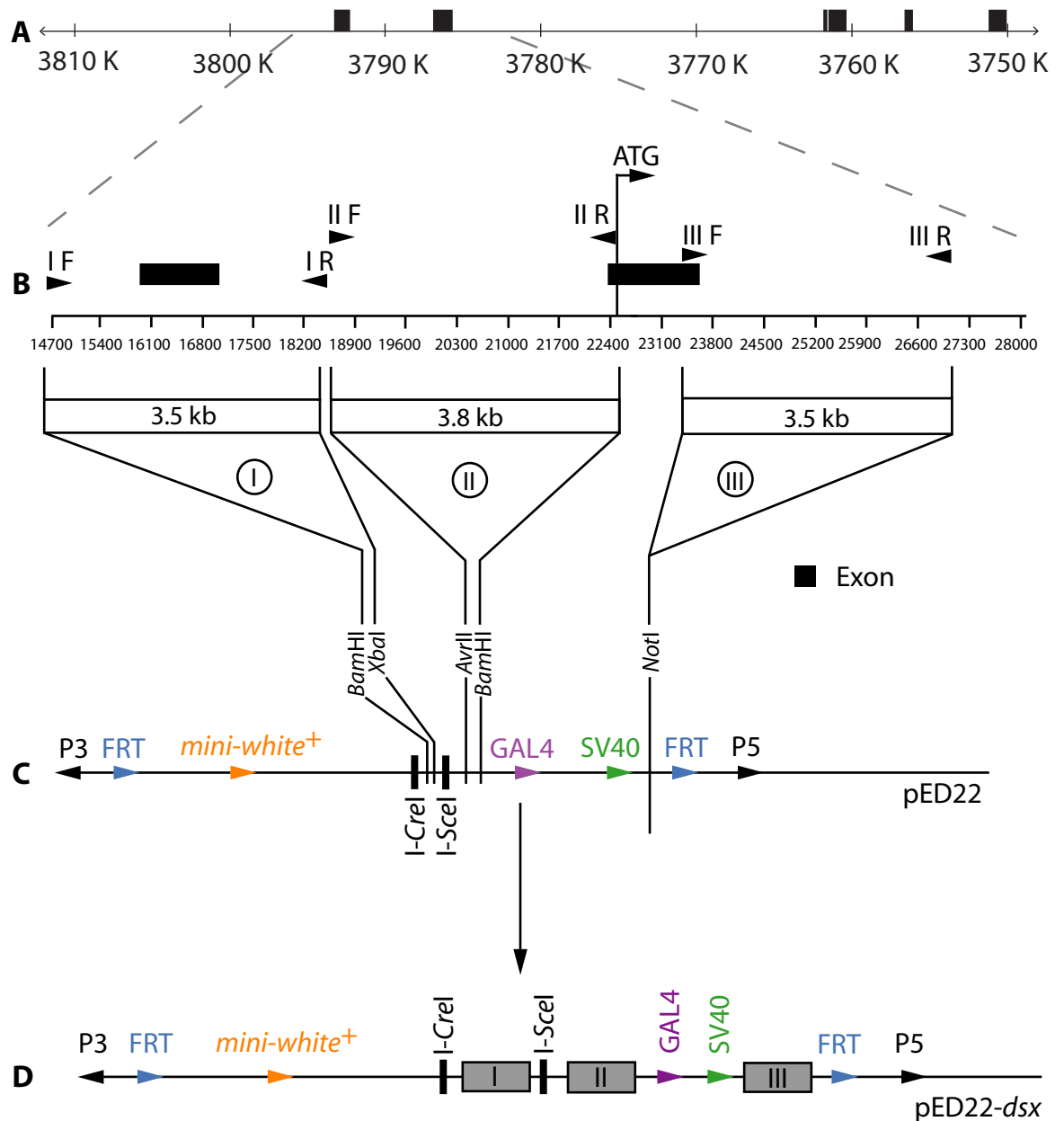
## 7.3 Generation of *dsx*<sup>GAL4</sup> Using Genetic Crosses

Once transformants were obtained with the pED22-*dsx* targeting vector integrated at random in the genome, the generation of *dsx*<sup>GAL4</sup> allele began, using genetic crosses to obtain the desired end product. Figure 7.2 shows the entire sequence of events required to generate the *dsx*<sup>GAL4</sup> allele in targeting the *dsx* gene by homologous recombination.

### 7.3.1 Integration

#### 7.3.1.1 Crossing Scheme and Number of Flies Crossed

Figure 7.3 shows the crossing scheme followed to obtain stable lines with the pED22-*dsx* vector integrated in the genome at the *dsx* locus. For the first cross, 150 virgin females were combined with 50 males in a bottle. The cross was left in the bottle for two full days, and then the adults were transferred into a new bottle on the morning of the third day. The bottle from which the flies were transferred was heat-shocked at 37°C for one and a half hours on the third day (just after flies were removed) and again on the fourth day for one hour. In total, 1900 virgin females were crossed, and were transferred into new bottles approximately ten times.



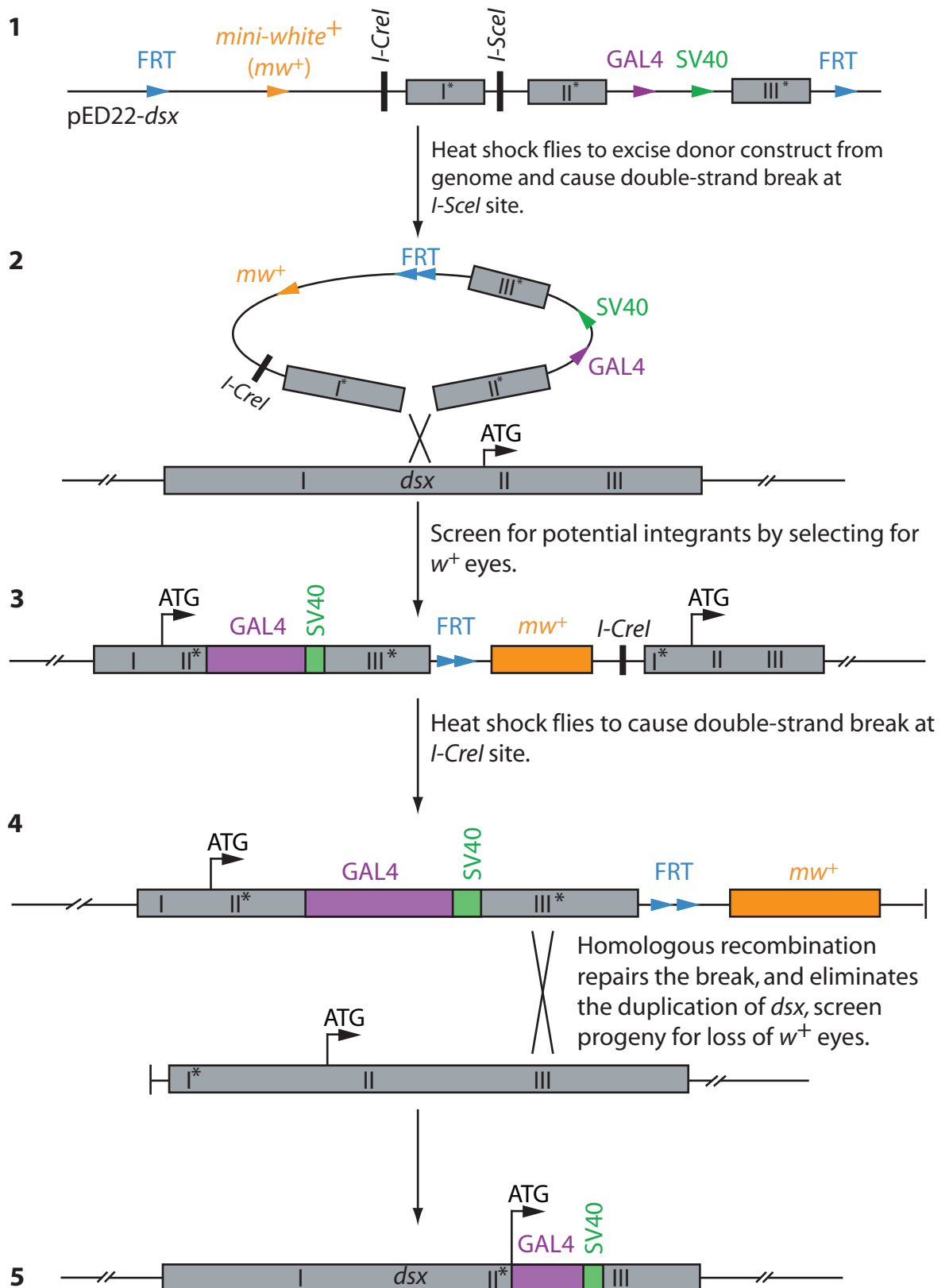
**Figure 7.1**-Cloning Strategy of Donor Construct for Ends-In Homologous Recombination. (A) Entire *dsx* locus from which regions of homology were isolated. Black boxes indicate exons. (B) Closer view of the region surrounding the first two exons, shown as black boxes, from which the fragments of homology were amplified. Forward (F) and reverse (R) primers for each of the three fragments of homology are shown. (C) Restriction sites incorporated into the forward and reverse primers allowed each fragment (sizes are shown) to be inserted into targeting vector pED22 (B. Dickson). (D) Final targeting construct, called pED22-*dsx*, is shown.

White-eyed, virgin females from this cross were collected in large numbers and the second cross was made as described in Figure 7.3 (progeny males from this cross were killed by a *hs-hid* transgene on the Y chromosome). Again, 150 females were combined with 50-70 males per bottle and allowed to lay eggs. After three to four days the adults were transferred into fresh bottles. In total, 8700 virgin females were crossed at this stage of the process, and were transferred into new bottles on average eight times.

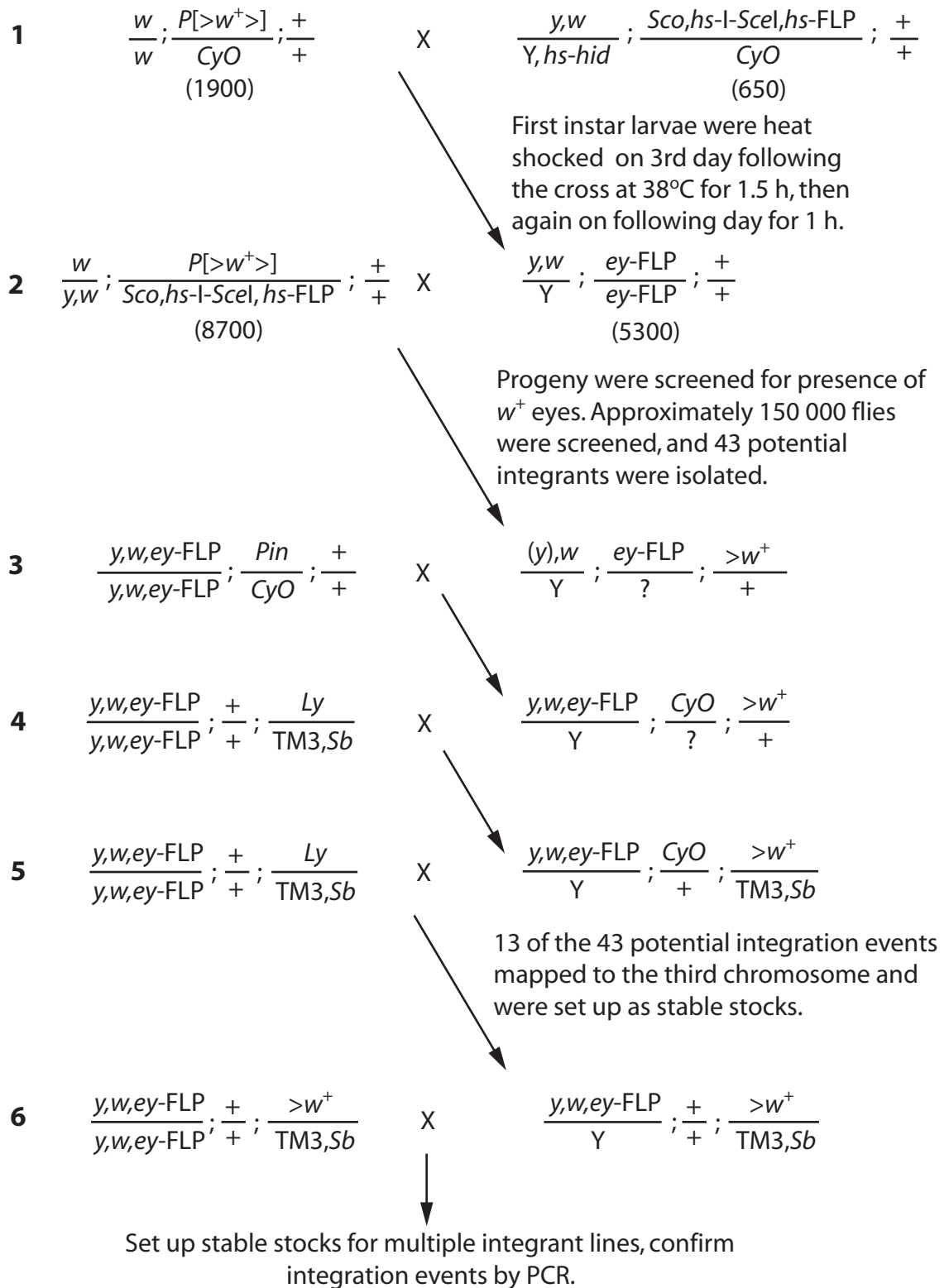
The progeny from this second cross were screened for the presence of orange eyes, to indicate the re-integration of the *mw*-containing targeting vector into the desired locus in the genome. In total, approximately 150000 flies were screened.

### 7.3.1.2 Relative Frequency of Episomal Integration

Of the 150000 flies screened, 43 were found with orange eyes. The eye colour was variable, some flies had solid orange eye colour, and others had a gradient of orange pigment across the eye (Figure 7.4). The orange-eyed flies fell into three categories, 1) orange pigment gradient across the eye (n=13), 2) solid orange colour (n=10), and 3) wild-type colour eyes (n=21). Crosses to map the chromosome with which the eye colour is associated found that only the flies with an orange gradient across the eye mapped to the third chromosome, where *dsx* is located, suggesting that only this class of eye colour represents an integration at the *dsx* locus. The flies with the wild-type eye colour mapped to the X chromosome, where the *w* gene is located; and together with the fact that many of these red-eyed flies were recovered from the same bottle (unlikely with rare targeting events), it seems most likely that this wild-type eye colour was a result of the contamination of one or two bottles with wild-type males when the adult flies were transferred between bottles. The solid orange eye colour was identical to the original *P*-element donor line, and given that this eye colour maps to the second chromosome, it is most likely due to the re-integration of the *P*-element donor at its original site of insertion. Therefore, the estimated frequency of integration into the *dsx* locus, based solely on the recovery of individuals with an orange pigment gradient across the eye, is approximately 1/11500 gametes.



**Figure 7.2-** Insertion of GAL4 into *dsx* Locus by Homologous Recombination. (1) 'Donor' construct pED22-*dsx* containing regions of homology I, II and III. Regions of homology originating from vector are marked with '\*'. (2) Donor construct is incorporated by homologous recombination into *dsx*, creating a duplication. (3) Orange-eyed integrants are identified and mapped to a chromosome. (4) A second double-stranded break is induced at the *hs-I-CreI* site, and repaired by homologous recombination to generate final allele (5) without the marker gene.



**Figure 7.3**-Genetic Crosses to Integrate Donor Construct into *dsx* Locus. (1) First instar larvae from the first cross are heat-shocked to kill male progeny, excise *P*-element donor and to make a double-stranded break in the donor, which is integrated into the target locus by homologous recombination. The number of flies crossed is indicated between parentheses. (2) Progeny from this cross are screened for presence of  $w^+$  gene marker. Number of flies crossed is indicated between parentheses. (3-5) Crosses to second and third chromosome balancers to map the chromosome of insertion of all independent  $w^+$  lines. (6) Individual  $w^+$  lines mapping to the correct chromosome are established as stocks, and subsequently the insertions are confirmed by PCR.

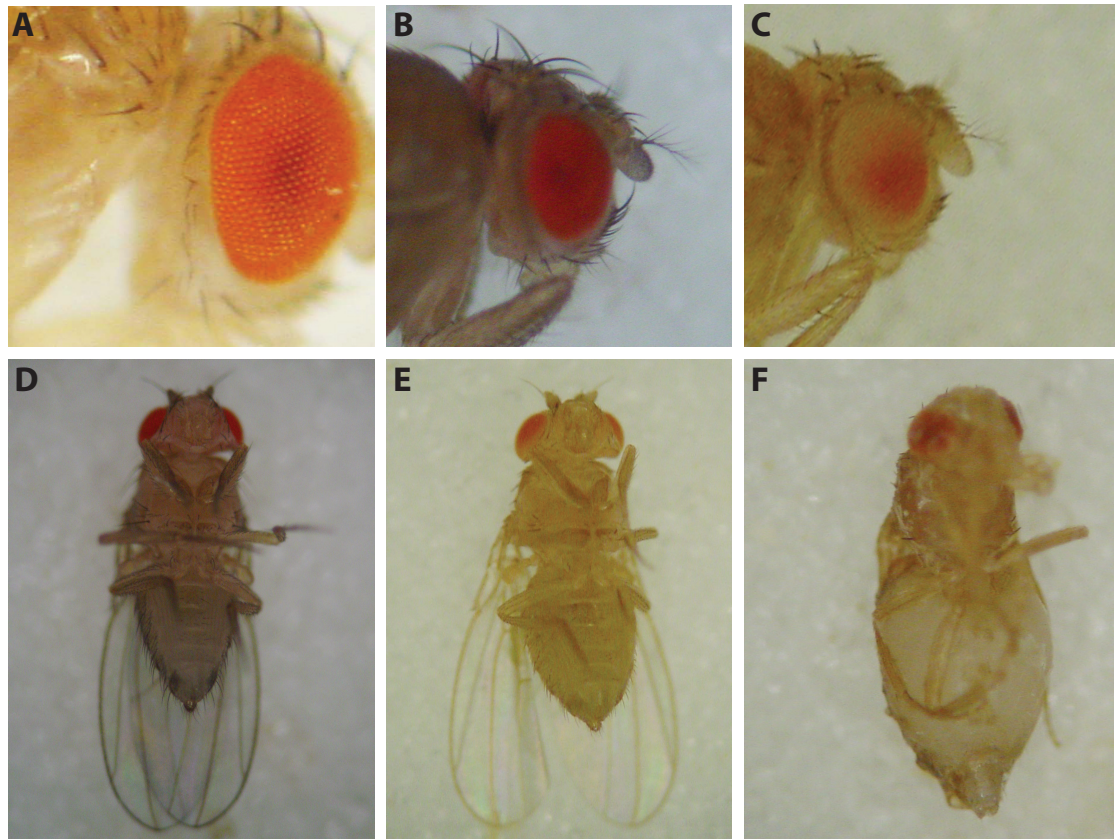
### 7.3.1.3 Verification of Integration by PCR

Primers were designed to confirm the FRT sites from the targeting vector came together during the initial *P*-element excision, and to verify the successful reinsertion of GAL4 into the genome. Genomic DNA was isolated from ten of thirteen lines (three lines have only a few individuals remaining), and the amplifications were performed to verify that these two events had occurred. Figure 7.5 shows the results from these PCR amplifications, and confirms that the FRT sites came together (shown in Figure 7.2) during the excision of the donor *P*-element from the genome in each of the potential integrant lines; and in addition, that each line contains GAL4. The original *P*-element donor line on the second chromosome was found to contain GAL4, as expected; and the PCR using the FRT primers failed to generate a product, as expected (Figure 7.5). *w<sup>1118</sup>*, the line into which the *P*-element targeting vector was microinjected, was used as a negative control for the amplifications with either the GAL4 or the FRT primers.

### 7.3.1.4 Phenotype of Integrant Flies

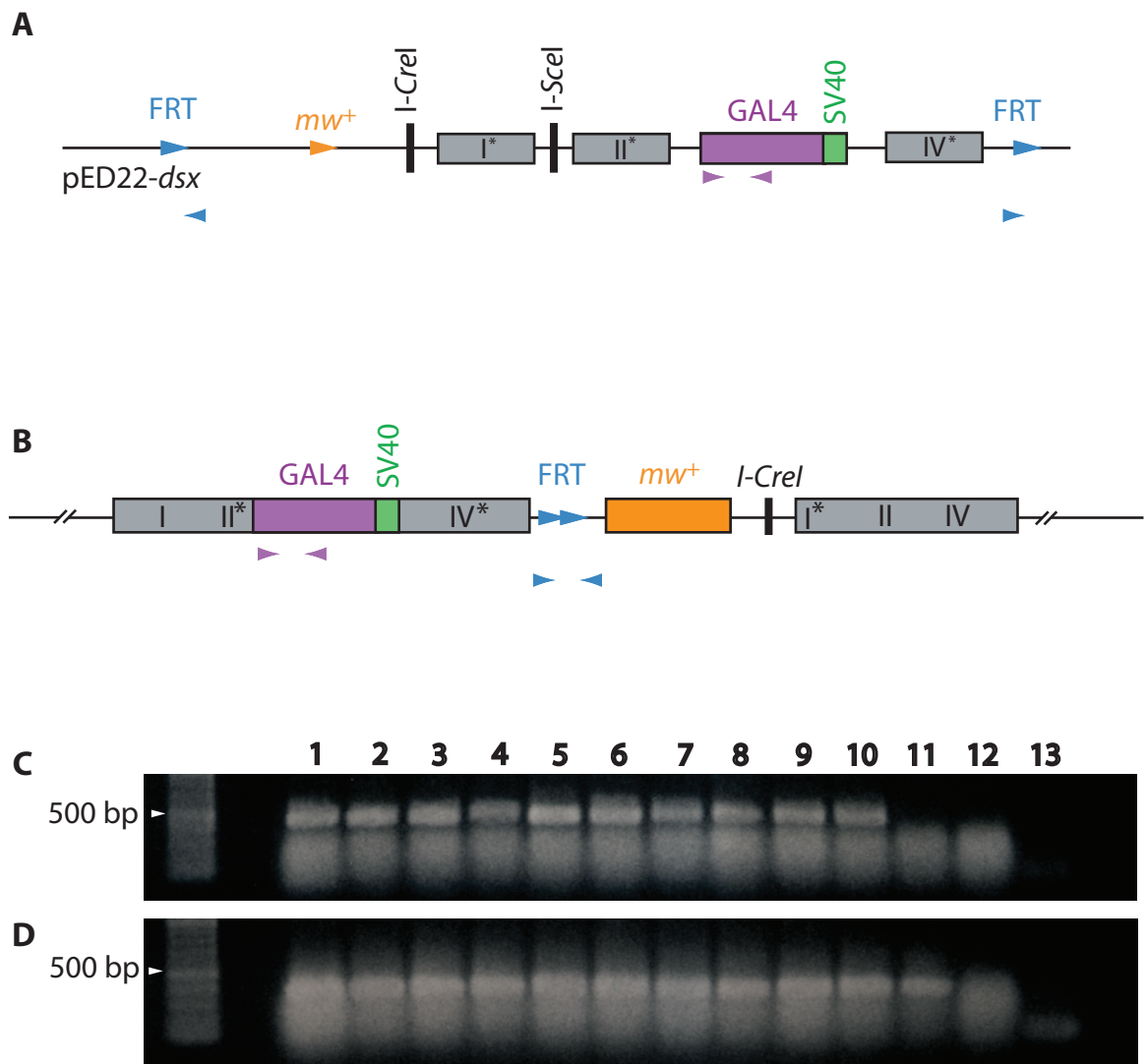
Flies containing the GAL4 insertion at the *dsx* locus were viable as homozygotes, and showed wild-type abdominal pigmentation and sex combs (in males). However, difficulties in maintaining the stock suggested that the duplication at the *dsx* locus caused fertility defects. Males and females heterozygous for this duplication were all fertile (n=18), as were males homozygous for the duplication (n=7); however, none of the females homozygous for the *dsx* duplication were fertile (n=13). The internal reproductive organs of these females were not abnormal, thus the reason for this infertility may be due to the retention of eggs, as suggested by the observation that the abdomens of these females become extremely distended over time (Figure 7.4F).

Together, these results confirm that the *P*-element donor has been excised from its original location on the second chromosome, that the *P*-element donor (as judged by mapping eye colour) is now located on the third chromosome, and that GAL4 is present in all lines. It therefore seems likely that the *P*-element donor has been successfully targeted to the *dsx* locus, creating a duplication. Thus, several duplication lines were used in the second stage of the gene targeting to resolve this duplication, and to create the *dsx<sup>GAL4</sup>* allele.



**Figure 7.4**-External Phenotype of Flies Containing a Duplication at the *dsx* Locus. (A) Near wild-type eye colour exhibited by several lines isolated during the screen for potential *P*-element donor integration events at the *dsx* locus. This eye colour always mapped to the X chromosome. (B) Lighter eye colour exhibited by several lines isolated during the screen for potential *P*-element donor integration events at the *dsx* locus. This eye colour always mapped to the second chromosome. (C) Eye colour exhibited by several lines isolated during the screen for potential *P*-element donor integration events at the *dsx* locus. This eye colour always mapped to the third chromosome, and thus likely represents *bona fide* integration at the *dsx* locus. Ventral view of the abdomen in females from (D) the original *P*-element donor line on the second chromosome, (E) heterozygotes for the integration at the *dsx* locus, and (F) homozygotes for the integration at the *dsx* locus.





**Figure 7.5**-Confirmation of Donor *P*-Element Excision and GAL4 Presence in the Genome by PCR. Primers are indicated by arrowheads, and direction is shown. (A) pED22-*dsx* donor construct is integrated randomly into the genome by *P*-element transformation. FRT primers (blue) are not oriented correctly to give a product, whereas the GAL4 primers (purple) will give a product of approximately 450 bp. (B) pED22-*dsx* integrated into the *dsx* locus, creating a duplication. Because the FRT sites have come together to form the circularized extrachromosomal plasmid in the first step of the gene targeting by homologous recombination, a product of approximately 500 bp is generated with the FRT (blue) primers. The GAL4 primers will also give a 450 bp product provided that GAL4 has been inserted intact into the *dsx* locus. (C) The PCR products from the amplification with the FRT primers were run on a 1% agarose gel and visualized with ethidium bromide on a shortwave UV transilluminator. (D) The PCR products from the amplification with the GAL4 primers were run on a 1% agarose gel and visualized with ethidium bromide on a shortwave UV transilluminator. All ten lines potentially carrying the *P*-element donor integrated at the *dsx* locus were screened, in addition to the original *P*-element donor line and a *w*<sup>1118</sup> negative control strain. (C,D) Lanes 1-13 were loaded as follows: (1-10) lines potentially carrying the integration at the *dsx* locus, (11) original *P*-element donor line on the second chromosome, (12) *w*<sup>1118</sup> negative control strain, (13) no DNA control. 100 bp DNA ladder (New England Biolabs) is shown on far left hand side of the gel.

### **7.3.2 Resolution**

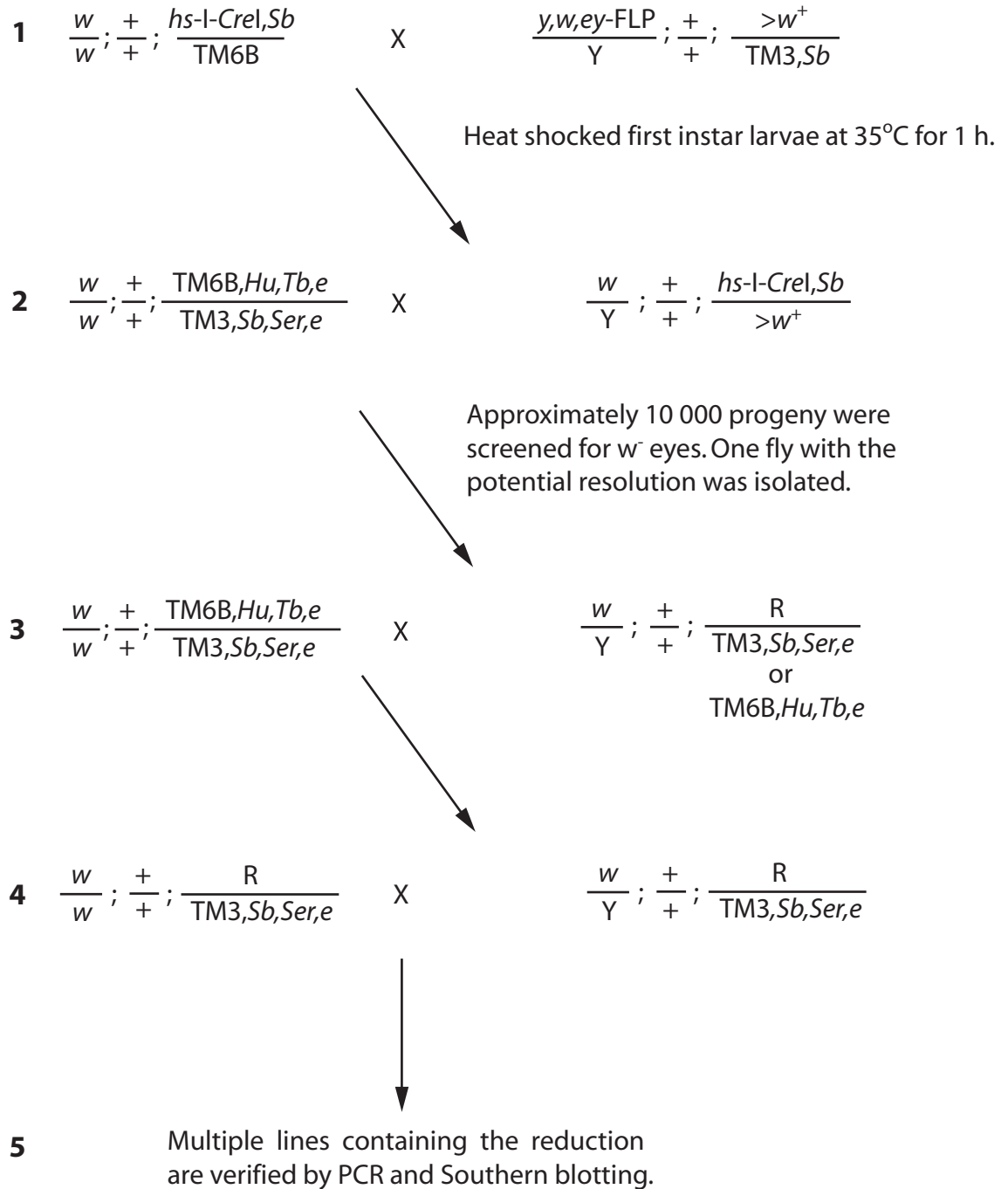
Thirteen independent lines were identified with a duplication of the *dsx* locus as a result of the integration of the donor *P*-element. The next step in the process is to resolve this duplication by inducing a double-strand break at the *dsx* locus, and eliminating the *mw* marker gene (Figure 7.2, Steps 3-5). The resolution of the duplication will be visible by the loss of the orange eye colour.

#### **7.3.2.1 Crossing Scheme and Number of Flies Crossed**

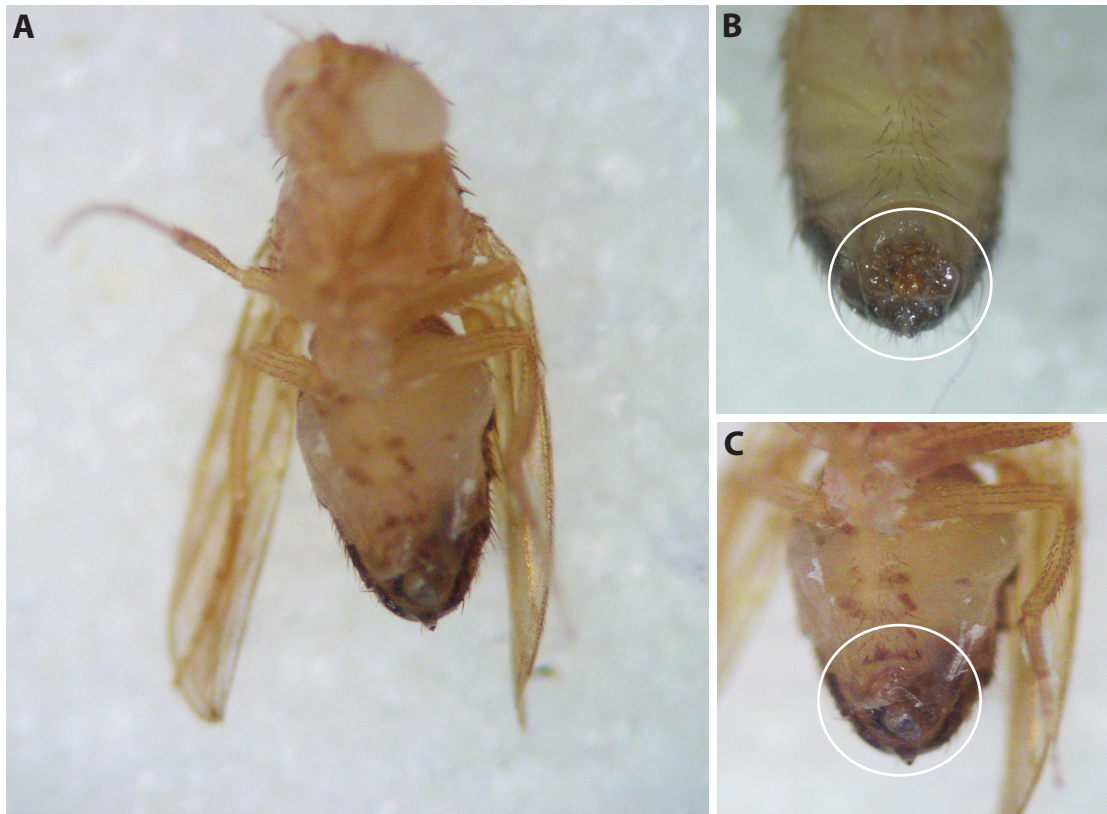
The crossing scheme for the second stage of gene targeting by homologous recombination is shown in Figure 7.6. Approximately 400 virgin females were crossed to males from each of six independent lines containing the duplication. Two days following the cross, the adults were transferred to new vials, and the old vials were heat shocked at 35-36°C for one hour. The adults from each vial were transferred on average five times each.

Approximately 50 male progeny from each duplication line with the appropriate genetic markers were collected and crossed to virgin females from a third chromosome balancer stock. Again, the adults in these vials were transferred on average ten times each.

Progeny from this cross were screened for the loss of the *mw* marker gene. From approximately 10000 flies screened, a single fly without the *mw* marker gene was found. Unfortunately, this single fly lacked distinguishable male or female genitals (Figure 7.7). The fly had extremely reduced sex combs, and male-like abdominal pigmentation; however, the absence of genitals prevented the continuation of the crosses required to stabilize, map and verify the elimination of the duplication by Southern blotting. Unfortunately, continued screening for flies lacking the *mw* marker gene identified no more such flies in the time remaining to the completion of this project, suggesting that resolution events at the *dsx* locus are fairly infrequent.



**Figure 7.6**-Genetic Crosses to Resolve the Duplication at the *dsx* Locus. (1) At least five independent lines containing the duplication at the targeted locus are crossed to the *hs-I-Crel* line, and the progeny of this cross are heat shocked as first instar larvae to induce a double-stranded break at the targeted locus. (2) Heat-shocked males carrying both *hs-I-Crel* and the duplication are crossed to a third chromosome balancer line, and the progeny screened for the loss of the  $w^+$  marker associated with the duplication. (3) Individuals lacking the  $w^+$  marker likely carry the resolved allele, designated 'R', and are singly crossed to females or males from a third chromosome balancer line. (4) Stable stocks for each of multiple independent lines potentially carrying the resolved allele are set up. (5) The presence of the resolved allele is verified by PCR and Southern blotting.



**Figure 7.7**-External Morphology of Individual Fly Containing the Resolved Allele of *dsx*. (A) Ventral view of the individual potentially containing the resolved allele of *dsx* called *dsx*<sup>GAL4</sup>. (B) Wild-type male external genitals indicated by white circle. (C) External genitalia of the fly potentially containing the resolved *dsx*<sup>GAL4</sup> allele, indicated by white circle.

## 7.4 Discussion

In this chapter, the process of gene targeting by homologous recombination was described, with the ultimate goal of inserting GAL4 into the *dsx* locus, and obtaining a new allele of *dsx* called *dsx*<sup>GAL4</sup>. Ideally, this new allele would drive expression of GAL4 in all *dsx*-expressing neurons, allowing the investigation of their structure, development and function. The results of these experiments are described in detail below.

### 7.4.1 Homologous Recombination

#### 7.4.1.1 Construct Design Criteria

The ends-in strategy of gene targeting by homologous recombination was used in this study used to insert GAL4 into the *dsx* locus. This strategy was used in a number of studies to make subtle alterations in a gene of interest, and was used successfully to insert GAL4 into the *fru* locus (Rong and Golic, 2000; Rong and Golic, 2001; Rong *et al.*, 2002; Stockinger *et al.*, 2005). Therefore, the construct to target the insertion of GAL4 to the *dsx* locus by ends-in homologous recombination was designed to ensure that the final *dsx*<sup>GAL4</sup> allele will be a *dsx* null mutant.

In order for the genetic basis of the anatomical, developmental and functional properties of sex-specific *dsx*-expressing neurons to be thoroughly examined, it must be possible to examine this novel *dsx*<sup>GAL4</sup> mutant allele *in trans* to extant *dsx* mutations, to investigate how Dsx expression influences the properties of these neurons. Previously, ends-in homologous recombination was used to insert the sequence for GAL4 into *fru*; however, as only the first step of the two-step process was completed, a large duplication remained at the locus. This *fru*<sup>GAL4</sup> allele (Stockinger *et al.*, 2005; Figure 3.1) was able to dictate the expression of GAL4 in all Fru<sup>M</sup> neurons; however, the presence of both the GAL4-containing and the wild-type exons at the *fru* locus led to the production of both GAL4-containing mutant transcripts and wild-type transcripts, giving a hypomorphic, rather than a null, mutant (Stockinger *et al.*, 2005). As a result, *fru*<sup>GAL4</sup> cannot be used *in trans* to extant *fru* mutants to investigate the contribution of Fru to the specification of the anatomical, developmental and functional properties of

*fru* neurons (Stockinger *et al.*, 2005). In this study, to avoid the production of a *dsx* hypomorphic phenotype, GAL4 was inserted into the *dsx* locus as described by Stockinger *et al.* (2005); however, the duplication was resolved in a second round of homologous recombination, to ensure the creation of a GAL4-containing *dsx* null allele.

#### **7.4.1.2 Ends-In vs. Ends-Out**

Ends-out homologous recombination allows the insertion of the donor targeting vector into a specific genomic location, and is often used to create null mutations in genes by simply disrupting either the enhancer regions or coding sequences of the desired gene. However, this type of disruption is not desirable when targeting genes with alternative splice forms, as disrupting the splicing may lead to the production of unexpected splice variants with unpredictable phenotypes. Manoli *et al.* (2005) described the successful insertion of GAL4 into the *fru* locus by ends-out homologous recombination; however, more recent work suggests that this insertion causes novel transcript classes of *fru* to be expressed in the CNS (Villella and Hall, pers. comm.). In addition, at the time these experiments were initiated, the vectors containing suitable genetic markers, along with loxP sites to remove the marker following the targeting, were not yet readily available. Therefore, the ends-in strategy of gene targeting by homologous recombination was used, as the targeting vectors were readily available, and the construct can be designed to ensure that a null mutant can be obtained with no disruption to *dsx*'s alternative splicing. Moreover, resolving the duplication allows the *mw* marker to be eliminated without engineering additional recombination sites into the targeting vector.

#### **7.4.2 Integration**

The first stage in the ends-in strategy was to simultaneously excise the *P*-element donor vector from its location in the genome and induce a double-stranded break in the construct to integrate the vector into the genome at the targeted locus by homologous recombination.

### 7.4.2.1 Frequency of Integration

The frequency of integration into the target locus can vary between loci, where the frequency of integration can range from 1 event in 1700 gametes to 1 event in 34000 gametes (Rong *et al.*, 2002). In this study the frequency of targeted events was found to be approximately 1 event in 11500 gametes, which falls within the range of frequencies reported for other loci. Studies on the frequency of targeted events in other systems and in *Drosophila* have found that the amount of donor:target homology can significantly increase the efficiency of targeting events more than fivefold (Rong *et al.*, 2002). Therefore, in future gene targeting experiments, larger regions of donor:target homology in the targeting vector will be used, to increase the efficiency of targeting, and reduce the number of flies to be screened.

### 7.4.3 Resolution

The second stage in the ends-in strategy was to induce another double-stranded break at the targeted locus using the site-specific I-*Crel* endonuclease, where the gene duplication is eliminated by homologous recombination. Six of the independent targeted alleles from the first stage of the process were used to resolve the duplication, as using multiple lines increases the likelihood of successfully obtaining the desired reduction at the gene of interest (E. Demir and B. Dickson, pers. comm.).

#### 7.4.3.1 Frequency of Resolution

Approximately 10000 flies in total were screened for the absence of the *mw* eye colour marker gene, to indicate that the desired reduction at the *dsx* locus had occurred. A single white-eyed fly was recovered from this screening, presumably containing the desired *dsx*<sup>GAL4</sup> allele. Thus the frequency of resolution is approximately 1 in 10000 gametes. In comparison to previous reports describing the reduction of the duplication of the targeted gene, this frequency of resolution is extremely low. Rong *et al.* (2002) reported that the average frequency of reduction to a single copy of the target genes *pugilist* (*pug*), *p53* (*Drosophila* homolog of *p53*), *Neural Lazarillo* (*NLaz*), *γ-glutamyl carboxylase* (*GC*) and CG11305 was 0.36. On the other hand, other laboratories have reported very low frequencies of resolution (B. Dickson, pers. comm.). In order

to improve the frequency of reduction/resolution at the *dsx* locus in the future, two further improvements to the strategy employed in this study are suggested: changing the the heat shock regime, and the *hs-I-Crel* line.

High levels of expression of the site-specific endonuclease I-Crel in *Drosophila* are likely to be lethal (Rong *et al.*, 2002). Unlike I-SceI, which has no recognition sites in the *Drosophila* genome and no discernible effects on viability, I-Crel is thought to have a cut site within the 28S ribosomal subunit gene, where 17 of 22 bp of this gene are identical to the recognition site for I-Crel, and this sequence is capable of being cut *in vitro* (Argast *et al.*, 1998). It is possible that too strong of a heat shock was used in this study, and the high levels of I-Crel expression induced high levels of lethality, decreasing the number of males surviving to reproduce in the first step of the resolution process (Figure 7.6). In future, a more moderate heat shock will be used to prevent this I-Crel induced lethality, and perhaps increase the frequency of resolution at the targeted locus.

Another possibility for the low frequency of flies containing the reduced copy of the targeted allele is the effectiveness of the *hs-I-Crel* transgenic line. Given the effects of *hs-I-Crel* on viability described above, it is possible that persistent levels of ectopic I-Crel expression from a potentially ‘leaky’ heat-shock promoter over time favours flies with extremely low levels of I-Crel expression. If this were true, over three years of keeping the stock prior to use, it is possible that changes in either I-Crel expression or the ability to induce I-Crel expression have accumulated, and that the transgene is no longer effective. To address this problem, a new *hs-I-Crel* line has been ordered from the *Drosophila* stock centre (Bloomington, University of Indiana), and will be used in all future experiments.

#### 7.4.3.2 Phenotype of Individual with a Resolved Duplication

A single fly potentially containing the reduced *dsx*<sup>GAL4</sup> allele was recovered, however, this individual lacked external genitalia, making it impossible to continue the genetic crosses required to stabilize and verify the novel *dsx* allele using genetic and molecular techniques. Given that a number of *dsx* null alleles are recessive (Hildreth, 1965; Duncan and Kaufman, 1975; Nothiger *et al.*, 1987; Baker *et al.*, 1991), it is surprising that the heterozygous combination of the novel *dsx* null allele should have such striking effects on the external reproductive structures of the fly isolated in this study. One possible explanation



is that the novel  $dsx^{GAL4}$  allele is dominant. Dominant alleles of  $dsx$  have been reported, where only the male-specific isoform of  $dsx$  is produced (Nothiger *et al.*, 1987; Baker and Wolfner, 1988; Nagoshi and Baker, 1990). In males, these dominant mutations do not affect either external or internal reproductive structures, or courtship behaviour (Baker and Ridge, 1980; Schupbach, 1982; Nagoshi and Baker, 1990; Taylor *et al.*, 1994). In females heterozygous for these dominant mutations ( $dsx^{Dom}/dsx^+$ ), the male isoform of  $dsx$  from the mutant allele is thought to compete on equal footing, or be dominant to the female isoform of  $dsx$  from the wild-type  $dsx$  allele, resulting in a partially masculinized female, with reduced sex combs and intersexual genitalia (Waterbury *et al.*, 1999). Females where the dominant allele is the only functional copy of  $dsx$  are transformed into pseudomales in appearance, but not in behaviour (Schupbach, 1982, Baker and Wolfner, 1988; Nothiger *et al.*, 1987; Taylor *et al.*, 1994). Therefore, it is possible that the isolated individual with the resolved allele of  $dsx$  was in fact a female heterozygous for a dominant mutation of  $dsx$ .

Could  $dsx^{GAL4}$  be a dominant mutation? In this study, the first coding exon of  $dsx$  was replaced by GAL4, along with the SV40 transcript terminator; however, the 3' end of the exon was left intact, to ensure that downstream splicing events, including sex-specific events, were not disrupted. Most dominant alleles of  $dsx$  have been reported to disrupt the female-specific splicing of  $dsx$  pre-mRNAs, resulting in the constitutive expression of the male isoform of  $dsx$  (Baker and Wolfner, 1988; Nagoshi and Baker, 1990). However, the alterations in this study were designed to ensure no disruption of the sex-specific splicing, thus it remains unclear how or why the resolved  $dsx^{GAL4}$  allele produced such striking effects on the external genitalia of the individual recovered.

## 7.5 Conclusions

In this chapter, the generation of a tool able to direct expression in all  $dsx$ -expressing cells was described. A targeting vector was constructed and inserted randomly into the genome by *P*-element transformation. Subsequently, this donor element was excised from the genome and integrated into the  $dsx$  locus, creating a duplication of the locus. Finally, the duplication was resolved and a fly with the first coding exon of  $dsx$  replaced with GAL4 was obtained; however, this  $dsx^{GAL4}$  chromosome was lost, as the single fly with this chromosome had no

identifiable external genitalia. It is possible that  $dsx^{GAL4}$  is a dominant mutation causing the constitutive production of the male-specific isoform of *dsx* in both sexes, and that the sole individual recovered in the resolution step of the gene targeting was in fact a female heterozygous for this dominant mutation. To resolve these questions, further attempts at resolving the duplication at the *dsx* locus will be made by screening on a large scale, where the sex of any individuals recovered, with mutant or wild-type genitalia, will be determined by performing single fly PCR using primers designed to amplify a Y-chromosome-specific gene, such as *kl-2* or *kl-3* (Carvalho *et al.*, 2000).

## **8 Discussion**

Overall, the aim of this work was to investigate the genetic and neurobiological factors underlying the sex-specific production of courtship song. Previous studies have shown that two sex determination genes, *fru* and *dsx*, are individually required for wild-type courtship song production (Ryner *et al.*, 1996; Villella and Hall, 1996; Villella *et al.*, 1997; Goodwin *et al.*, 2000). The work presented in this thesis extended these findings by showing that both genes are required for the production of courtship song, suggesting that *fru* and *dsx* act either in parallel or in concert to specify song production. An investigation of the co-expression of *fru* and *dsx* in the CNS revealed the co-expression of Fru<sup>M</sup> and Dsx in three distinct regions, including the Msg, a region of the CNS strongly implicated in the production of courtship song (von Schilcher and Hall, 1979). A detailed examination of the Msg subsequently identified a sexually dimorphic population of Fru<sup>M</sup>-expressing neurons in this region, whose presence was associated with the ability to generate wild-type courtship song. Significantly, both Fru<sup>M</sup> and Dsx<sup>M</sup> were required for the specification of this sexually dimorphic population of neurons in the Msg. Together, these results suggest that both *fru* and *dsx* are required in the specification of a neural substrate critical to the production of courtship song; however, a number of additional questions were raised:

- Why are both *fru* and *dsx* required to specify the sexually dimorphic production of courtship song?
- What is the significance of the differences created by *fru* and *dsx*?

In this chapter, these questions will be discussed with regard to the results presented in this thesis, and programme of experiments to resolve these outstanding questions will be proposed.

## **8.1 Why are both *fru* and *dsx* required to specify the sexually dimorphic production of courtship song?**

Both *fru* and *dsx* were found to be required for the specification of courtship song, but also in the creation of a sexually dimorphic population of neurons in the Msg. Individually, each gene is capable of creating sexually dimorphic neuronal populations elsewhere in the CNS (Taylor and Truman, 1992; Kimura *et*

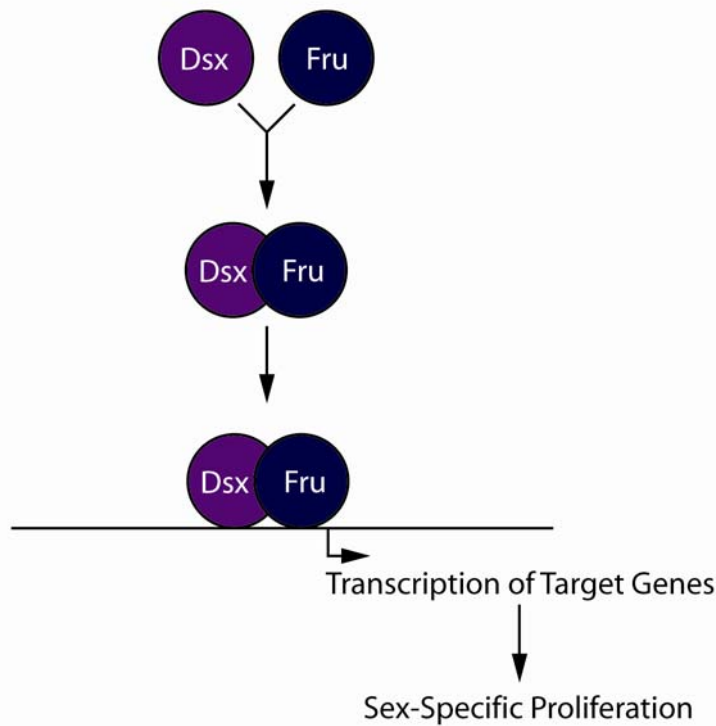
*al.*, 2005); however, in the *Msg*, a sexually dimorphic population of neurons is obtained only when the male-specific isoforms of each gene are present, suggesting that *fru* and *dsx* act together to create this population of neurons. However, there are a number of ways in which two genes can act ‘together’ to specify a sexually dimorphic developmental programme.

### **8.1.1 Do *fru* and *dsx* act in concert, in parallel, or consecutively?**

In Chapter 5, the co-expression of Fru<sup>M</sup> and Dsx in three distinct regions of the CNS was shown. Given the requirement for both genes in courtship song, it is interesting that Fru<sup>M</sup> and Dsx are co-expressed in the *Msg*, a region known to be important for song production. Moreover, the specification of a sexually dimorphic population of neurons in the *Msg* linked to song production requires the expression of both Fru<sup>M</sup> and Dsx. The significance of this co-expression is not yet known, but three explanations for the presence of Fru<sup>M</sup> and Dsx in the same neurons are the following: 1) Fru<sup>M</sup> and Dsx physically interact and bind together to downstream targets to specify a common programme of sex-specific developmental events, 2) Fru<sup>M</sup> and Dsx work in parallel in the same neurons, where the sex-specific developmental events specified by each are mutually exclusive, but converge upon a common process, or 3) Fru<sup>M</sup> and Dsx act consecutively in the same neurons during development, where the sex-specific developmental events specified by each are temporally distinct but complementary.

#### **8.1.1.1 In Concert?**

Fru<sup>M</sup> and Dsx are both transcription factors, and as described above, the requirement for both Fru<sup>M</sup> and Dsx in the specification of a population of sexually dimorphic neurons in the *Msg* could be a product of a direct interaction between the two proteins, where this interaction is required in order to activate sex-specific downstream targets and pathways (Figure 8.1).



**Figure 8.1**-Schematic Representation of a Model in Which Dsx and Fru<sup>M</sup> Directly Interact. Schematic representation of a model in which the underlying cause of the requirement for both *dsx* and *fru* in the specification of a sexually dimorphic CNS is a result of a direct interaction between the two proteins. In this model, Fru and Dsx bind to one another, and then act together to regulate genes involved in the regulation of proliferation and/or cell death.

Fru<sup>M</sup> proteins are sequence specific transcription factors with C2H2 zinc finger DNA binding domains, and a BTB protein-protein interaction domain (Ryner *et al.*, 1996; Ito *et al.*, 1996). Studies have shown that the BTB domains often form homodimers (Albagli *et al.*, 1995; Dhordain *et al.*, 1995); however, heterodimers with BTB domains from other proteins are also possible (Bardwell and Treisman, 1994; Hoatlin *et al.*, 1999; Kobayashi *et al.*, 2000). To date, no Fru<sup>M</sup> interacting proteins have been published; however, a yeast two-hybrid screen has identified a number of candidates (M. Neville, pers. comm.).

Dsx is a transcription factor with an amino-terminal DM DNA binding domain, and a protein interaction domain at its carboxy-terminus (Burtis and Baker, 1989; Burtis *et al.*, 1991; Erdman and Burtis, 1993). The fundamental binding species of both the male- and female-specific isoforms of Dsx is as a dimer, however, Dsx contains oligomerization domains, and can form oligomers in higher protein concentrations (An *et al.*, 1996; Cho and Wensink, 1996). Given that the physical

characteristics and DNA binding by the sex-specific isoforms of Dsx are virtually indistinguishable, it has been proposed that the specificity in function arises as a result of sex-specific protein-protein interactions (Cho and Wensink, 1996; Cho and Wensink, 1997). Indeed, the function of the female-specific isoform of Dsx as a transcription factor has been shown to depend on an interaction with putative transcription factor *intersex* (*ix*) (Garrett-Engle *et al.*, 2002), demonstrating that sex-specific interactions can be responsible for aspects of sexually dimorphic development.

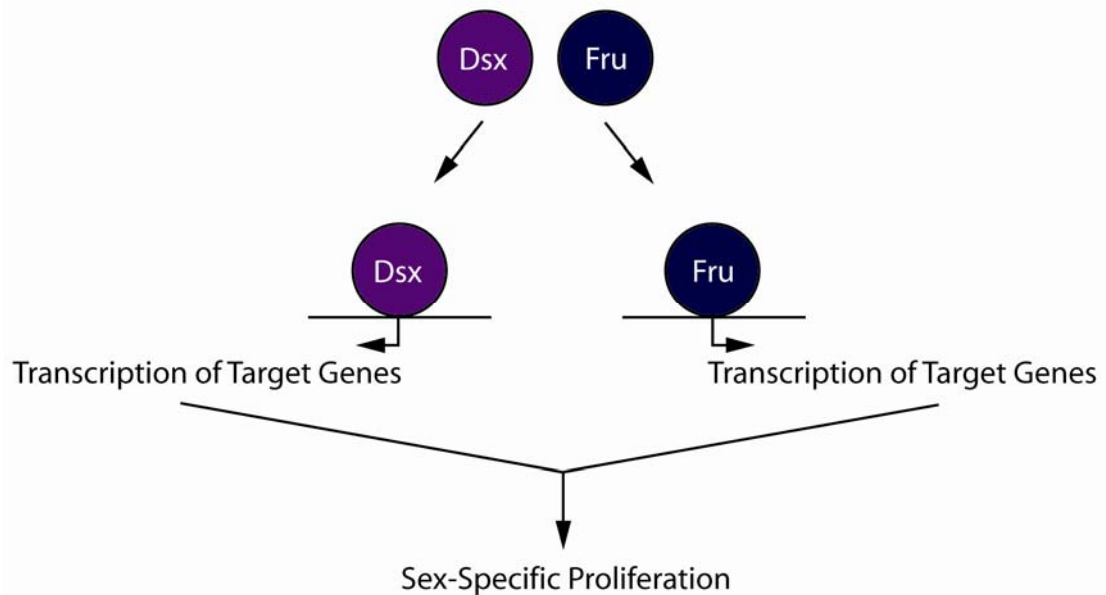
To investigate whether the requirement for both *fru* and *dsx* in the specification of sexually dimorphic neuronal populations is a result of a direct interaction between the two proteins, a number of experimental approaches can be taken to determine whether the two proteins physically interact. As a first step, the yeast two-hybrid system would be ideal to determine whether the two proteins are capable of interacting *in vitro*. This system fuses full-length cDNAs from *fru* and *dsx* either to the activation or the binding domain of yeast transcriptional activator GAL4. When the two plasmids are co-transformed into a modified yeast strain, if the two proteins interact, they bring the activation and binding domains of GAL4 into close proximity, and the GAL4 binds to and activates a reporter gene(s) integrated into the host genome (Giot *et al.*, 2003; reviewed by Parrish *et al.*, 2006). If an interaction is detected in the yeast two-hybrid system, the next step to further verify this interaction would be to co-immunoprecipitate the two proteins from *Drosophila* S2 cell lines, or from whole fly protein extracts. In 2002, Garrett-Engle *et al.* co-transfected constructs capable of expressing tagged versions of either Dsx<sup>F</sup> or Dsx<sup>M</sup>, along with a tagged version of *ix*, into *Drosophila* S2 cells. Monoclonal antibodies against the tagged *ix* protein were then used to recover the tagged *ix* from the cell lysate, and the immunoprecipitates and supernatants were resolved by SDS-PAGE and detected by Western blotting with antibodies against either the tagged Dsx<sup>F</sup> or Dsx<sup>M</sup>. Only the tagged Dsx<sup>F</sup> protein, not Dsx<sup>M</sup>, was shown to co-immunoprecipitate with the tagged *ix* protein (Garrett-Engle *et al.*, 2002). A similar approach could therefore be taken to confirm the interaction of Fru<sup>M</sup> and Dsx, by generating tagged constructs of each gene, and creating transgenic lines by *P*-element transformation. The lines containing these constructs could then be crossed, and the co-immunoprecipitation of the two proteins from tissues of interest would proceed as described above (Garrett-Engle *et al.*, 2002).

The use of a combination of the techniques described above would allow the investigation of whether *fru* and *dsx* physically interact, and further experiments could confirm whether this interaction is required to activate downstream transcriptional targets. However, the fact that Fru<sup>M</sup> is capable of creating a sexually dimorphic population of neurons in a region of the CNS where it is not co-expressed with Dsx suggests that Fru<sup>M</sup> alone is sufficient in some cases to trigger the cascade of events required for the development of sex-specific neuronal populations (Kimura *et al.*, 2005). Moreover, the expression of Dsx<sup>M</sup> alone, in the absence of Fru<sup>M</sup>, is sufficient to prolong neuroblast divisions in the Abg to create a sex-specific population of neurons (Taylor and Truman, 1992), again suggesting that the function of either Dsx<sup>M</sup> or Fru<sup>M</sup> is not dependent on a physical interaction with the other. Finally, studies have shown that Dsx proteins can bind to their only molecularly confirmed targets, the *yolk protein (yp)* genes, in the absence of Fru<sup>M</sup> (Burtis *et al.*, 1991; Coschigano and Wensink, 1993; Erdman and Burtis, 1993; Cho and Wensink, 1996; Cho and Wensink, 1997), suggesting that at the molecular level, the functions of Fru<sup>M</sup> and Dsx do not depend on a physical interaction. Therefore, the requirement for both genes in the specification of a sexually dimorphic neuronal population in the Msg is not likely due to a requirement for a direct interaction.

#### 8.1.1.2 In Parallel?

In the absence of a physical interaction between Fru<sup>M</sup> and Dsx, another possibility for the requirement for both genes in the specification of a sexually dimorphic CNS is that the downstream targets of Fru<sup>M</sup> and Dsx are different, however, the targets activated or repressed by either Fru<sup>M</sup> and/or Dsx contribute towards a common process to generate a sexually dimorphic CNS, such as neurogenesis (Figure 8.2).





**Figure 8.2**-Schematic Representation of a Model in Which Dsx and Fru<sup>M</sup> Act in Parallel During Development. Schematic representation of a model in which the underlying cause of the requirement for both *dsx* and *fru* in the specification of a sexually dimorphic CNS is the parallel but complementary pathways activated independently by *dsx* and *fru*. In this model, *fru* and *dsx* have mutually exclusive downstream target genes, which are activated independently, but ultimately function in a complementary manner to achieve proliferation (or cell death).

Prior to investigating whether Dsx and Fru<sup>M</sup> act in parallel in the CNS to specify a sexually dimorphic CNS, the mechanism by which these sexually dimorphic populations of neurons are created would have to be known, which will be discussed in more detail in 8.1.3. However, once the mechanism by which these sex-specific neuronal populations are created is known (provided it is a single mechanism), the UAS/GAL4 system of targeted gene expression can be used to investigate the requirement for each gene in the neurons in which they co-localize. For example, flies containing *fru*<sup>GAL4</sup>, a driver line which expresses in all *fru* neurons (Stockinger *et al.*, 2005), could be crossed to flies carrying a UAS-*dsx* inverted repeat (IR) construct (Vienna Drosophila RNAi Centre). Expressing inverted repeats of a gene in *Drosophila* generates double-stranded RNA homologous to the gene of interest, which triggers the degradation of the endogenous mRNA, resulting in a loss of the transcript, and hence the protein product of the gene of interest (Fire *et al.*, 1998; reviewed by Carthew, 2001; Montgomery, 2004; Kavi *et al.*, 2005). In this way, the effect of losing Dsx expression only in Fru<sup>M</sup> neurons on the developmental process responsible for generating sex-specific neurons can be determined. Then, the reciprocal experiment using the *dsx* promoter-GAL4 fusion *dsx*(15)-GAL4, or *dsx*<sup>GAL4</sup>, in

combination with UAS-*fru*-IR, could be performed. If the sexually dimorphic neuronal populations were created by the convergence of the two pathways on sex-specific neurogenesis, for example, then the loss of either Dsx or Fru<sup>M</sup> in those sex-specific neurons would lead to defects in neurogenesis, resulting in fewer neurons in these regions in males. However, as described in section 8.1.1.1, Dsx and Fru<sup>M</sup> alone can each specify the creation of sexually dimorphic neuronal populations, making it less likely that separate developmental pathways triggered by each gene are required to converge on a single process for generating a sex-specific CNS.

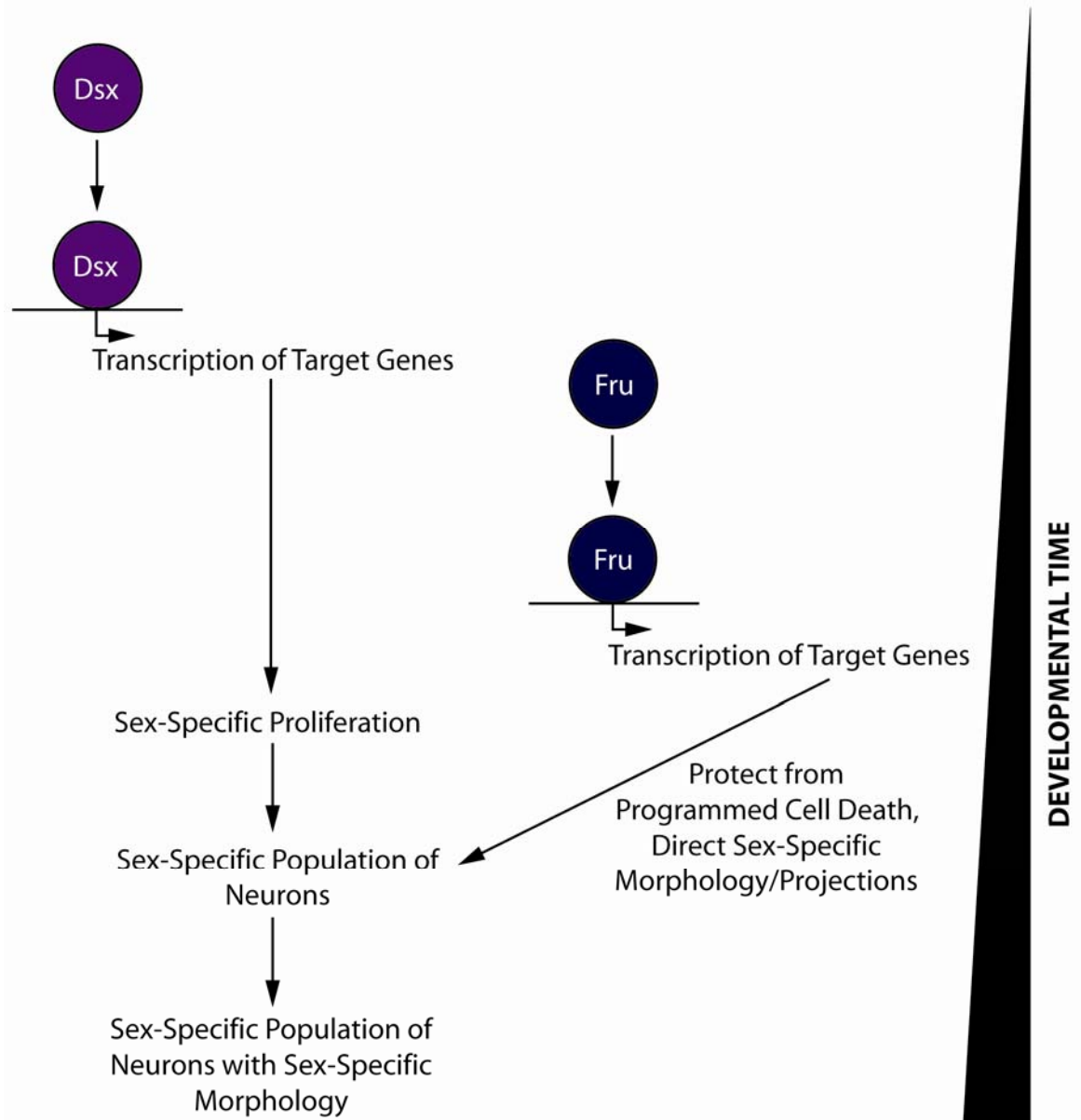
### 8.1.1.3 Consecutively?

A final explanation for the dual requirement for *fru* and *dsx* in the specification of sexually dimorphic CNS is that the developmental pathways initiated by each of the two genes are separated in time, but are complementary to one another (Figure 8.3). This possibility seems the most likely, as each gene in this model is sufficient to initiate a given process, which is supported by the experimental evidence; however, the combination of the two distinct processes can lead to a third outcome. The other models require that Fru<sup>M</sup> and Dsx be able to independently direct the sex-specific development of the CNS in some regions, but to be depend on one another in other regions, which is possible, but less likely.

Dsx<sup>M</sup> directs the creation of a male-specific population of neurons in the Abg by prolonging neuroblast divisions (Taylor and Truman, 1992). Fru<sup>M</sup>, on the other hand, has been shown to create a male-specific population of neurons in the brain by preventing programmed cell death in the neurons in which it is expressed (Kimura *et al.*, 2005). In addition, the expression of Fru<sup>M</sup> in these neurons is also responsible for specifying their sex-specific neuronal morphology (Kimura *et al.*, 2005). Although Fru<sup>M</sup> and Dsx are co-expressed in the CNS in 2-day-old pupae and 5-day-old adults, and both are expressed at the highest levels in 2-day-old pupae, Dsx expression slightly precedes Fru<sup>M</sup> expression in development, suggesting that the action of Dsx may precede the action of Fru<sup>M</sup> (Lee *et al.*, 2000; Lee *et al.*, 2002). Provided this were true, it is possible to imagine a situation where Dsx prolongs the neuroblast divisions in specific regions of the CNS, to generate a sex-specific population of neurons, which are

then either protected from programmed cell death and/or directed to develop a sex-specific morphology by Fru<sup>M</sup>. To test this hypothesis, the number of neurons expressing a reporter driven by the *dsx(15)*-GAL4 line, which expresses in most Dsx neurons in the posterior brain, could be counted first in wild-type males and females, and then in both *fru* and *dsx* mutant backgrounds, at various points during development. If the number of neurons expressing the reporter increased in a sex-specific way, it would suggest that sex-specific neurogenesis was involved. Moreover, if this sexually dimorphic proliferation was absent in *dsx* mutant males, it would suggest that *dsx* controlled the sex-specific neurogenesis. If these neurons then showed a sex-specific decrease in number following the initial increase, it would suggest that sex-specific programmed cell death was responsible for enhancing the initial differences created by sexually dimorphic neurogenesis. Performing this experiment in *fru* mutants would allow the role of *fru* in this process to be evaluated. Then, the projections of these sex-specific neurons could be examined in both wild-type and *fru* and/or *dsx* mutant backgrounds using the UAS-mCD8::GFP reporter (Lee and Luo, 1999), to determine whether the projections or axonal morphology of the neurons are affected by either Fru<sup>M</sup> or Dsx expression.

The experiments described in 8.1.1 will allow the nature of the requirement for both *fru* and *dsx* in the specification of a sexually dimorphic neuronal population to be determined. In 8.1.3, experiments to identify the mechanism by which these neuronal populations are created will be discussed in further detail.

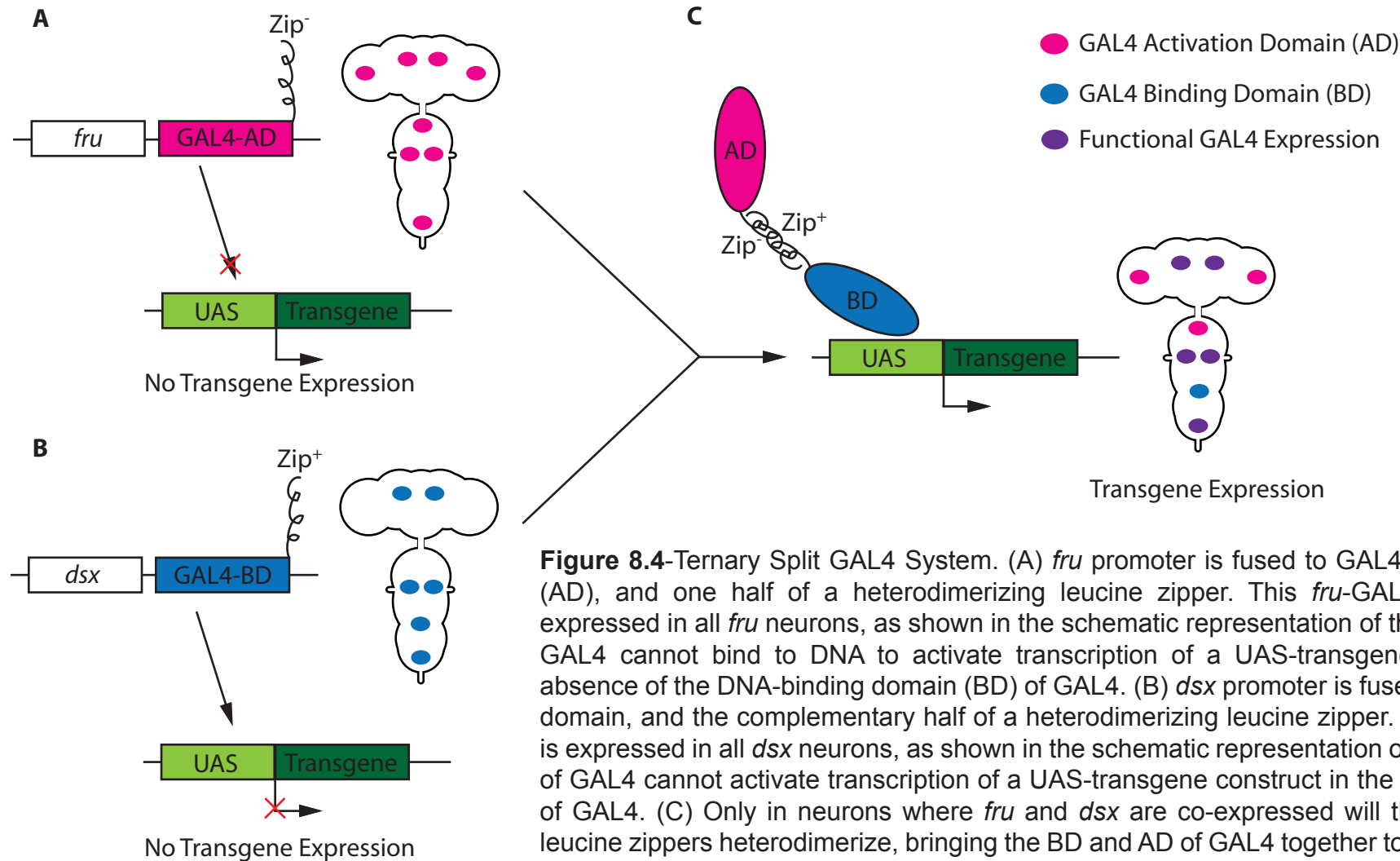


**Figure 8.3**-Schematic Representation of a Model in Which *Dsx* and *Fru*<sup>M</sup> Act Consecutively in Development. Schematic representation of a model in which the underlying cause of the requirement for both *dsx* and *fru* in the specification of a sexually dimorphic CNS is the consecutive actions of both genes in the CNS to generate sexually dimorphic neuronal populations. In this model, *dsx* activates (or represses) transcriptional targets early in development, which then act to generate a sex-specific population of neurons in the CNS. Then, *fru* activates (or represses) transcriptional targets later in development, which are expressed in these sex-specific neurons, and protect them from cell death.

### 8.1.2 *Dsx* in Non-Neuronal Tissues vs *Dsx* in the CNS

In Chapters 3 and 5, a requirement for both *fru* and *dsx* in the specification of a sexually dimorphic cluster of neurons in the *Msg* linked to the production of courtship song was shown. However, the requirement for both *fru* and *dsx* was shown using *tra* mutant females, which are chromosomal females transformed into pseudomales as a result of the constitutive expression of *Dsx<sup>M</sup>*. Although *Dsx<sup>M</sup>* plays a critical role in the creation of a sexually dimorphic cluster of neurons in the CNS, its strong effect on behaviour and courtship song could be at least partially due to the masculinization of other tissues in addition to the CNS. Previous studies have shown that the feminization of the fat body in male flies with UAS-*tra* led to a decrease in the amount of courtship performed by these males (Dauwalder *et al.*, 2002). Moreover, it was recently shown that the amount of courtship behaviour performed by the *fru<sup>M</sup>* and *fru<sup>Δtra</sup>* females was significantly improved when the fat body was also ‘masculinized’ (Lazareva *et al.*, 2007). Together, these results demonstrate an important role for a tissue outside the CNS in the control of sexual behaviour. Given that *dsx* affects sexual differentiation in many tissues outside the CNS, a technique must be found to distinguish the contribution of *dsx* expression to the sexual differentiation of non-neuronal tissues from its contribution to sexual differentiation in the CNS, to determine whether the critical role of *dsx* in the specification of courtship behaviour and song production is mediated by its expression exclusively within the CNS, or whether other tissues are involved.

Recently, a ternary split GAL4 system was developed to allow the expression of a UAS-reporter/transgene construct only in cells where the expression patterns of two GAL4 drivers intersect (Figure 8.4; Luan *et al.*, 2006). Luan *et al.* (2006) split GAL4 into its component parts: an activation domain (AD), and a DNA binding domain (BD), where each component was cloned downstream of a different promoter. Neither of these ‘hemidrivers’ on its own can mediate the activation of a UAS-reporter/transgene; however, in cells where the expression driven by the two promoters intersects, the components of GAL4 are brought together to form a functional transcriptional activator, capable of driving the expression of a UAS-reporter/transgene (Figure 8.4).



**Figure 8.4-Ternary Split GAL4 System.** (A) *fru* promoter is fused to GAL4 activation domain (AD), and one half of a heterodimerizing leucine zipper. This *fru*-GAL4-AD construct is expressed in all *fru* neurons, as shown in the schematic representation of the CNS. The AD of GAL4 cannot bind to DNA to activate transcription of a UAS-transgene construct in the absence of the DNA-binding domain (BD) of GAL4. (B) *dsx* promoter is fused to GAL4 binding domain, and the complementary half of a heterodimerizing leucine zipper. The *dsx*-GAL4-BD is expressed in all *dsx* neurons, as shown in the schematic representation of the CNS. The BD of GAL4 cannot activate transcription of a UAS-transgene construct in the absence of the AD of GAL4. (C) Only in neurons where *fru* and *dsx* are co-expressed will the complementary leucine zippers heterodimerize, bringing the BD and AD of GAL4 together to activate transcription of the UAS-transgene construct. Adapted from Luan *et al.* (2006).

This ternary split GAL4 system would be an ideal way to examine the contribution of *dsx* in the CNS to the specification of sexual behaviour, by first inserting the GAL4 AD into *dsx* by homologous recombination, and then using this construct in conjunction with a line where *elav*, a neuron-specific marker (Yao *et al.*, 1993), is fused to the GAL4 BD (Luan *et al.*, 2006). In this way, the behavioural consequences of a variety of experiments targeting only *dsx*-expressing neurons can be determined, using an assortment of UAS constructs, such as UAS-*dsx*-IR (to knock down Dsx expression), UAS-*shi*<sup>ts</sup> (to reversibly disrupt neuronal function), and UAS-*hid* (to ablate the neurons). In addition, if the GAL4 AD is inserted into other loci, similar experiments can be performed to investigate the behavioural consequences of disrupting *dsx* expression (or *dsx*-expressing cells) exclusively in the fat body or the gustatory neurons on the male foreleg, for example. Ultimately, these experiments will determine the significance of Dsx expression in the CNS in the specification of behaviour, and how the function of *dsx*-expressing neurons in the CNS contributes to the generation of sex-specific behaviours. Moreover, the role of Dsx expression in tissues outwith the CNS can be determined, to establish how Dsx expression in these tissues contributes to the specification of courtship behaviour.

### **8.1.3 How Are Sex-Specific Neuronal Populations Created in Regions of Co-Expression?**

A common feature of the regions in which *fru* and *dsx* are co-expressed is the creation of sex-specific populations of neurons. In the previous section, three models were proposed to explain why both *fru* and *dsx* are required for the specification of these sexually dimorphic neuronal populations. In this section, the possible mechanisms by which these sex-specific populations are generated will be discussed, along with experiments to distinguish between the possibilities.

#### **8.1.3.1 Developmental Mechanisms**

Different developmental pathways can be exploited in order to achieve a male- and a female-specific CNS, such as sex-specific neuronal proliferation and/or sex-specific programmed cell death. Neurogenesis and programmed cell death occur throughout development, from embryonic stages up to the first day

postmetamorphosis, to shape the adult CNS (Truman and Bate, 1988; Kimura and Truman, 1990; Truman, 1990; Abrams *et al.*, 1993; White *et al.*, 1994; Grether *et al.*, 1995; Chen *et al.*, 1996). Both sex-specific neurogenesis and programmed death have been reported (Truman and Bate, 1988; Taylor and Truman, 1992; Kimura *et al.*, 2005), demonstrating the importance of these two mechanisms in generating a CNS equipped to perform all the stereotypical behaviours of that sex.

## Neurogenesis

Postmitotic neurons are generated in *Drosophila* by the symmetrical division of a cell called a ganglion mother cell (GMC), which is the smaller cell generated by the asymmetric division of a neuroblast (reviewed by Jan and Jan, 2001; Chia and Yang, 2002; Betschinger and Knoblich, 2004). Following mitotic divisions during embryonic development, neuroblasts in the VNC become quiescent until they begin a series of postembryonic divisions during larval and pupal stages to generate the adult CNS (Prokop and Technau, 1991). A few neuroblasts in the Abg undergo postembryonic neurogenesis (Truman and Bate, 1988), where these neuroblasts have been shown to depend on the expression of  $Dsx^M$  to continue dividing (Taylor and Truman, 1992). Thus  $Dsx^M$ -mediated neurogenesis may be the mechanism by which sex-specific neuronal populations in regions of the CNS where  $Fru^M$  and  $Dsx$  are co-expressed are generated.

Traditionally, BrdU incorporation is used to label actively dividing neuroblasts in the CNS, and the number of dividing neuroblasts in a given region of the CNS can be determined and compared between the sexes. This was the technique used by Taylor and Truman (1992) to study the sex-specific divisions of neuroblasts in the Abg, where only a few neuroblasts undergo postembryonic divisions; however, in other thoracic ganglia, the majority of the embryonic neuroblasts undergo postembryonic divisions, making the comparison of a small population of neuroblasts using BrdU incorporation between the sexes virtually impossible. Thus another marker to reveal neuroblasts and/or actively dividing neurons must be found. Recently, Cornbrooks *et al.* (2006) showed that of the 24 thoracic adult-specific lineages arising from larval thoracic neuroblasts (Truman *et al.*, 2004), Delta expression specifically identifies 7 lineages. Delta is a ligand with both an extra- and intracellular domain that signals to neighbouring cells



through Notch receptors, mediating cell fate decisions and neurite outgrowth (reviewed by Portin, 2002). Elevated Delta expression was found in newly generated neurons adjacent to distinct clusters of neuroblasts/GMCs; interestingly, Delta was expressed in the male-specific neurons of the Abg generated by the prolonged neuroblast divisions in males (Cornbrooks *et al.*, 2006). Thus to determine whether the sex-specific population of neurons in the Msg is generated by sexually dimorphic neurogenesis, Delta expression in the Msg can be compared in males and females, to identify any differences in the number of neurons generated by each of the 7 Delta-expressing lineages. Moreover, Delta expression in the Msg of *dsx* and *fru* mutant males can also be examined, to determine the role of these genes in specifying sexually dimorphic neurogenesis in the Msg.

Although investigating Delta expression in the Msg may reveal sex-specific neurogenesis, the sexually dimorphic population of neurons in the Msg may not be included in one of the seven Delta-expressing lineages. Another marker which labels neuroblasts in the thoracic ganglia of the CNS in *Drosophila* is the sequence specific DNA-binding protein Grainyhead (Grh) (Bray *et al.*, 1989; Dynlacht *et al.*, 1989; Bray and Kafatos, 1991; Attardi and Tijan, 1993; Uv *et al.*, 1994; Uv *et al.*, 1997; Brody and Odenwald, 2000; Bello *et al.*, 2003). An antibody against Grh can therefore be applied to the CNS of both males and females (Bray *et al.*, 1989), to determine if a dimorphism in the number of thoracic (or abdominal) neuroblasts exists. Then, it can be determined whether Grh-expressing neuroblasts also express either Fru<sup>M</sup> or Dsx, to investigate whether these two genes might be involved in controlling neuroblast proliferation. Finally, using *dsx(15)*-GAL4, *dsx*<sup>GAL4</sup> or *fru*<sup>GAL4</sup>, the number of Grh-expressing neuroblasts can be counted in *fru* and *dsx* mutant males, to determine whether fewer neuroblasts are present in these mutants in comparison to wild-type males.

Still another approach to investigating the *fru*- and *dsx*-mediated creation of sexually dimorphic neuronal populations would be to use *dsx(15)*-GAL4 to drive the expression of a reporter in the Dsx-expressing neurons in the posterior brain, to investigate neurogenesis this region. These neurons are sex-specific, therefore, the reporter expression driven by *dsx(15)*-GAL4 can be co-localized with BrdU-labelled neuroblasts using anti-BrdU antibodies. This is not possible in

the *Msg*, as the driver has no expression in *Dsx* neurons in this region, and *Dsx* itself requires very gentle fixation conditions incompatible with those required to detect BrdU-labelled neurons with anti-BrdU (Truman and Bate, 1988). Therefore, although these experiments will first be performed in the posterior brain, once the *dsx*<sup>GAL4</sup> allele is isolated by homologous recombination, it will be possible to extend this analysis to the *Msg*.

If these sex-specific neurons in the posterior brain or in the thoracic ganglia are found to co-localize with BrdU, or any of the other neuroblast markers described in this section, it will show that these sex-specific neurons are in fact neuroblasts, and demonstrate that neurogenesis plays a critical role in the generation of a sexually dimorphic CNS. Moreover, these experiments will demonstrate the mechanism by which *Fru*<sup>M</sup> and *Dsx*<sup>M</sup> generate a sex-specific CNS, capable of dimorphic behaviours.

As discussed in 8.1.1.3, given what is known about *Dsx*<sup>M</sup>'s role in the sexual differentiation in the CNS, it seems probable that sex-specific neurogenesis mediated by *Dsx*<sup>M</sup> is at least partly responsible for the creation of sexually dimorphic neuronal populations (Taylor and Truman, 1992); however, the next section will discuss the potentially complementary role of programmed cell death alongside sex-specific neurogenesis in generating these dimorphic populations of neurons.

## Cell Death

Cell death genes *grim*, *rpr* and *hid* are all required for programmed cell death in *Drosophila*, where cell death is promoted by the activation of caspase pathways (White *et al.*, 1994; Hay *et al.*, 1994; Bump *et al.*, 1995; Grether *et al.*, 1995; Chen *et al.*, 1996; White *et al.*, 1996; McCarthy and Dixit, 1998; Haining *et al.*, 1999). Recently, two studies by DeFalco *et al.* (2003) and Kimura *et al.* (2005) showed that sex-specific apoptosis in male-specific gonadal precursors (msSGPs) and the CNS, respectively, were under the control of genes of the sex determination pathway. The msSGPs, which eventually become part of the testis, are eliminated in females during development by *Dsx*<sup>F</sup>-promoted programmed cell death (DeFalco *et al.*, 2003). In the CNS, a cluster of neurons in the brain in females is eliminated during development by programmed cell death, where the expression of *Fru*<sup>M</sup> in males protects this cluster of neurons

from programmed cell death (Kimura *et al.*, 2005). Thus both *fru* and *dsx* have roles in either promoting or preventing cell death to create sexually dimorphic structures in *Drosophila*.

To investigate whether sex-specific programmed cell death of neurons contributes to the creation of the sexually dimorphic neuronal populations in regions where Fru<sup>M</sup> and Dsx are co-expressed, the MARCM system for making mosaics will be used. The MARCM method employs heat shocks delivered at different developmental times to induce mitotic recombination, which will ‘mark’ cells dividing at that time and all progeny cells with a reporter gene such as GFP (Lee and Luo, 1999; reviewed by Duffy, 2002). In addition, the ‘marked’ cell can be made mutant for a given gene, allowing the impact of that mutation on cell survival or morphology to be determined (Lee and Luo, 1999; reviewed by Duffy, 2002). Therefore, by inducing mitotic recombination at different developmental times, in males and females, the Dsx neurons in which *dsx(15)*-GAL4 drives expression can be made mutant for any combination of cell death genes *grim*, *rpr*, or *hid*, to investigate which of the genes (if any) are involved in creating sexually dimorphic populations of neurons in this region. If cell death is involved in the creation of this population of neurons, then the individual and combined roles of *fru* and *dsx* in promoting cell death can also be assayed using the MARCM system, by observing the survival (or not) of these neurons when they are made mutant for *fru*, *dsx*, or both.

In addition to programmed cell death in postmitotic neurons of the CNS, neuroblasts also undergo programmed cell death, to end neural proliferation, and control the final number of progeny neurons generated by an individual neuroblast (Peterson *et al.*, 2002; Bello *et al.*, 2003). In the abdominal ganglion of the CNS, the expression of Hox protein Abdominal-A (AbdA) induces programmed cell death in neuroblasts to limit the number of progeny they generate; whereas in the thoracic ganglia of the CNS, neuroblasts do not normally die as early, and generate many more progeny per neuroblast (Bello *et al.*, 2003; Cenci and Gould, 2005). However, these studies do not distinguish between male and female specimens, thus any dimorphisms in neuroblast cell death may have been overlooked (Bello *et al.*, 2003). To investigate whether this is the case, it is possible to co-localize Grh (to mark a neuroblast) with either *Antennapedia* (*Antp*) or *Ultrabithorax* (*Ubx*), two Hox genes present in the

thorax which are capable of activating programmed cell death in neuroblasts (Bello *et al.*, 2003; Cenci and Gould, 2005), to determine whether neuroblasts in females undergo early programmed cell death, ultimately generating fewer neurons in the thoracic ganglia of the CNS. Alternatively, *Ubx* and *Antp* expression in the thoracic ganglia can also be compared between the sexes, to find any regions in which sex-specific cell death may be activated.

Yet another possible approach is to investigate the expression of Polycomb group proteins in neuroblasts in the thoracic region of the CNS in male and females. Recently, Bello *et al.* (2007) showed that Polycomb group proteins were required for neuroblast survival and proliferation in the CNS. Therefore, by comparing Polycomb group expression in males and females, it can be determined whether any dimorphisms are present, which would in turn suggest that female neuroblasts undergo early apoptosis, ultimately generating fewer neurons than in the male thoracic CNS. In addition, if a dimorphism in Polycomb group or Hox protein expression is found, the roles of *fru* and *dsx* in the creation of these differences can be investigated by co-localizing Fru<sup>M</sup> and Dsx with the Polycomb group or Hox proteins, and by examining the expression of Polycomb group or Hox proteins in *fru* and *dsx* mutants.

Together, these experiments will give insight into the role played by cell death, either of neurons or of neuroblasts, in generating a sexually dimorphic CNS. Given that Fru<sup>M</sup> is able to protect neurons from programmed cell death in other regions of the CNS (Kimura *et al.*, 2005), it seems possible that it plays a similar role throughout the CNS; however, whether it is involved in neuroblasts survival remains to be seen. As discussed in section 8.1.1.3, and given the known roles of Fru<sup>M</sup> and Dsx in creating sexually dimorphic populations of neurons in the CNS, it seems most likely that Fru<sup>M</sup> and Dsx act consecutively during development, where Dsx generates a sexually dimorphic population of neurons by sexually dimorphic neurogenesis, and Fru<sup>M</sup> ensures survival and potentially specifies the morphology of the sex-specific neurons (or neuroblasts).

#### **8.1.3.2 When are *fru* and *dsx* required?**

Both *fru* and *dsx* are required to create sexually dimorphic neuronal populations, likely by exploiting either sex-specific neurogenesis or programmed cell death. However, both neurogenesis and programmed cell death occur throughout

development, from embryonic stages to metamorphic and into early adult stages (Truman and Bate, 1988; Kimura and Truman, 1990; Truman, 1990; Abrams *et al.*, 1993; White *et al.*, 1994; Grether *et al.*, 1995; Chen *et al.*, 1996). When do *fru* and *dsx* exert their critical influence on these processes?

Dsx expression is first detected in third instar larvae, peaks during metamorphosis, and continues at low levels into adulthood (Lee *et al.*, 2002). Similarly, Fru<sup>M</sup> expression is first detected in late third instar larvae, peaks during metamorphosis, and continues into adulthood (Lee *et al.*, 2000). Using a temperature sensitive allele of the yeast protein GAL80, which has been shown to inhibit GAL4 activity, it is possible to investigate the temporal requirement for either *fru* or *dsx*. GAL80 binds to GAL4 and prevents GAL4-mediated transcriptional activation, and has no deleterious phenotypic effects in flies (Ma and Ptashne, 1987; Lee and Luo, 1999). Using a ubiquitously expressed, temperature sensitive allele of GAL80 (McGuire *et al.*, 2003), GAL4-mediated transcriptional activation in *fru* or *dsx* mutants can be inhibited until a heat-shock is given at different points in development. In this way, the expression of UAS-*fru* or UAS-*dsx* under the control of *fru*<sup>GAL4</sup>, *dsx*(15)-GAL4, or *dsx*<sup>GAL4</sup> can be controlled during development so that the time each gene is required for the development of sex-specific neuronal populations can be determined.

### **8.1.4 Conclusions**

Together, the experiments described above will allow a greater understanding of the requirement for *fru* and *dsx* in the specification of sexually dimorphic neuronal populations in the CNS, and in addition, contribute to the understanding of the mechanism by which these differences are created.

## **8.2 What is the significance of the differences created by *fru* and *dsx*?**

In the previous section, the creation of sex-specific differences by *fru* and *dsx* was discussed. In this section, the functional implications of these differences will be discussed, along with a series of experiments to reveal the significance of a sexually dimorphic CNS.

### **8.2.1 How Does the Absence of a Few Neurons Cause Such Striking Effects on Song?**

The focus of this thesis has been the investigation of the genetic and neurobiological factors underlying the sexually dimorphic nature of courtship song. A sexually dimorphic population of neurons in the *Msg* was identified, and implicated in the production of courtship song. Yet how does the absence of these neurons result in the highly aberrant song phenotype of *dsx* mutants and *Fru<sup>M</sup>*-expressing females?

#### **8.2.1.1 Development**

One possible reason that the absence of the male-specific population of neurons in the *Msg* causes such striking song defects could be that the cluster of neurons acts as a target or guide for other neuronal populations during development. In the absence of these target/guide neurons, other clusters of neurons may not develop the stereotypical morphology or connections required for courtship song. Recently, Truman *et al.* (2004) used the MARCM method to identify 24 adult-specific thoracic lineages arising from larval thoracic neuroblasts, and found that the pathfinding decisions of neurons from a given lineage were extremely consistent, and projected to a shared location. Most often, the target of the projections from a single lineage appeared to be the neurons from another lineage, which led the authors to suggest that the connectivity of the adult CNS may develop from these early between-lineage connections (Truman *et al.*, 2004). Thus, the absence of a cluster of neurons could potentially affect the pathfinding ability of other neural clusters, ultimately leading to an alternative connectivity in females or mutants lacking the male-specific cluster of neurons in the *Msg*.

Although the elegant study by Truman *et al.* (2004) to identify the early projection patterns of neurons of each of 24 lineages per hemisegment provides a map of the early connectivity of the larval thoracic ganglia, it is not stated in the study whether any of the lineages show sex-specific characteristics. Therefore, the first step in determining whether the early connectivity of the thoracic ganglia is sexually dimorphic is to use the MARCM system to compare the projections of specific *fru*- or *dsx*-expressing lineages in males and females

(as in Truman *et al.*, 2004). The MARCM system can then be used to investigate the contributions of *fru* and *dsx* to the development of these (if any) sex-specific projections in a given lineage by making GFP-marked *fru* or *dsx* mutant lineages in male individuals.

### 8.2.1.2 Function

Although the sexually dimorphic population of neurons in the CNS may be critical during development to establish the early connectivity of the CNS, the function of these sex-specific neurons must also contribute to the ability to perform sex-specific behaviours such as courtship song. Therefore, to investigate the function of these sex-specific neurons, the UAS/GAL4 system of targeted gene expression can be used to disrupt their function, and the courtship song of these individuals can be analyzed. Several issues need to be addressed before this type of experiment can be performed, including the following: 1) a GAL4 line(s) expressing in *dsx* neurons is required, and 2) the disruption must only occur in the adult CNS to ensure development is not perturbed.

In this study, two GAL4 lines have been made: one with expression in the body and *dsx*-expressing neurons in the posterior brain (*dsx(15)*-GAL4), and another potentially expressing in all *dsx*-expressing cells (*dsx*<sup>GAL4</sup>). These two lines can therefore be used to investigate the behavioural implications of disrupting the function of the sex-specific neurons in the posterior brain, the Msg and the Abg. In this experiment, the temperature sensitive allele of *shi* (*shi*<sup>ts</sup>; Kitamoto, 2001) will be used to reversibly disrupt the function of these neurons. Other transgenes are available which are capable of disrupting neuronal function, such as UAS-*Kir2.1* or UAS-*dORK4*, which electrically silence neurons by reducing the probability of action potential firing (Baines *et al.*, 2001; Nitabach *et al.*, 2002). However, these other transgenes disrupt neuronal function throughout development, and are not reversible, and therefore are not ideal to investigate neuronal function only in adults.

Given that both *dsx(15)*-GAL4 and *dsx*<sup>GAL4</sup> have expression outwith the CNS, however, an additional level of control is required to ensure that only neuronal function is disrupted. Stockinger *et al.* (2005) generated a UAS>FRT>STOP>FRT>*shi*<sup>ts</sup> construct, in which the expression of *shi*<sup>ts</sup> is limited by the restricted expression of FLP, which excises the STOP from the UAS to permit

*shi<sup>ts</sup>* expression. In this proposed experiment, *dsx(15)*-GAL4 or *dsx<sup>GAL4</sup>* containing *elav*-FLP could be crossed to a line containing UAS>FRT>STOP>FRT>*shi<sup>ts</sup>*, so that *shi<sup>ts</sup>* expression would be restricted to *dsx(15)*-GAL4- or *dsx<sup>GAL4</sup>*-expressing neurons in the CNS. Then, courtship song quality could be analyzed in individuals at both the restrictive and permissive temperatures, and the behavioural and song defects of individuals examined using *dsx(15)*-GAL4 or *dsx<sup>GAL4</sup>* could be compared, to determine the function of specific clusters of sexually dimorphic neurons in generating courtship song and behaviours.

Given that sex-specific neuronal populations are created in regions in which Fru<sup>M</sup> and Dsx are co-expressed, another way in which the functional significance of these sex-specific neurons can be investigated is by using the ternary split GAL4 system of targeted gene expression (Luan *et al.*, 2006). As shown in Figure 8.4, the AD of GAL4 can be inserted into the *fru* locus, and the GAL4 BD can be inserted into the *dsx* locus by homologous recombination. Then, only the neurons in which Fru<sup>M</sup> and Dsx are co-expressed will express a transgene, for example UAS-*shi<sup>ts</sup>* (Kitamoto, 2001; Luan *et al.*, 2006). In this way, the contribution of this male-specific ‘circuit’ created by *fru* and *dsx* to the specification of sexual behaviour can be investigated.

A final approach to understanding the function of this putative male-specific circuit is to use the ternary split GAL4 system to express an optically-gated ion channel only in the neurons in which Fru<sup>M</sup> and Dsx are co-expressed. Then, a pulse of light will activate this subset of neurons, where the flies may be stimulated to perform sex-specific courtship behaviours. Using *fru<sup>GAL4</sup>* to express this optically-gated ion channel in decapitated males, it has been shown that activating only all thoracic *fru* neurons is sufficient to trigger the song motor pattern in males (G. Miesenböck, pers. comm.). Thus, by isolating small clusters of sex-specific neurons using the ternary split GAL4 system, it can be determined whether certain subsets of neurons are both necessary and/or sufficient for a given behaviour, by selectively activating or disrupting neuronal function.

### 8.2.1.3 Circuitry

The work presented in this thesis has identified sexually dimorphic neuronal populations in two regions of the CNS in which Fru<sup>M</sup> and Dsx are co-expressed, the Msg and the posterior brain; other studies have identified the sexually



dimorphic neurons in the Abg, the third region of Fru<sup>M</sup> and Dsx co-expression (Truman and Bate, 1988). Previous sections have discussed possible explanations for the dual requirement for *fru* and *dsx* in the specification of these neurons, and proposed experiments to further investigate how sexually dimorphic neuronal populations develop, their impact on the sex-specific development of the CNS, and the contributions of individual clusters to the specification of specific behaviours. In this section, however, the relationship between these sexually dimorphic neuronal clusters will be discussed.

A recent study by Billeter *et al.* (2006b) showed that the fertility defects exhibited by males mutant for the Fru<sup>M</sup> isoform containing zinc finger C (Fru<sup>MC</sup>), could be rescued by using a GAL4 line expressing only in a subset of all Fru<sup>M</sup> neurons. This experiment demonstrates that all male-specific behaviours are not likely controlled by a single ‘male behavioural circuit’; instead, specific regions of the CNS can be responsible for subsets of male behaviours. However, some co-ordination or communication between these individual neural networks must be required to integrate the performance of these male behaviours into the observed stereotypical sequence.

Given that the expression of *fru* and *dsx*’s male-specific isoforms in a female is sufficient to induce the performance of male behaviours in these females, the creation of sexually dimorphic populations in regions of co-expression suggests that these sex-specific neurons form at least part of this co-ordinating circuit. In this way, some neurons in these clusters may play an essential role in a given behaviour, but other neurons in the same cluster may form essential connections with other clusters, to communicate information from one region of the CNS to other regions. When these connections are not present, the information is not transmitted, and behaviour is disrupted or absent. For example, females do not normally perform courtship behaviour, therefore, they may require no specialized connections between olfactory regions of the CNS, which detect pheromones, and the thoracic ganglia, which produce the motor output for courtship song and attempted copulation. In males, on the other hand, communication between olfactory centres and motor output centres is critical to ensure that sensory stimulation triggers an appropriate behavioural response.

To determine whether any direct connections exist between the sexually dimorphic populations of neurons, the stereotypical projection pattern of the neurons in each cluster will have to be investigated. Using the MARCM method, the projections of single neurons or clusters of neurons in these sex-specific clusters will be marked using UAS-mCD8::GFP (Lee and Luo, 1999). If these preparations are also incubated with anti-Dsx, it can be determined whether the projections of the sex-specific neurons in the *Msg* project to the Dsx-expressing dimorphic population of neurons in the posterior brain, or alternatively, the *Abg*. Any direct connections between these clusters would suggest that one of the critical roles of these sexually dimorphic neurons is to establish communication between regions of the CNS responsible for individual behaviours, to ensure they are performed in a co-ordinated manner. Moreover, if these connections between different regions of the CNS were abolished in the absence of *fru* or *dsx*, it would give critical insight into how the action of two genes can create the potential for sexually dimorphic behaviour in *Drosophila*.

### 8.3 Conclusions

In this study, the neurobiological basis for the sexually dimorphic production of courtship song in *Drosophila melanogaster* was investigated; in addition, the roles of two sex determination genes *fru* and *dsx* in generating some of these essential differences were examined. Differences in the number of neurons in the *Msg* between males and females were found, a region of the CNS strongly implicated in courtship song production (von Schilcher and Hall, 1979). Both *fru* and *dsx* were found to be required to create these differences, and the absence of the sex-specific neurons was correlated with song defects. In the future, the mechanism by which *fru* and *dsx* specify the creation of these dimorphisms will be investigated, and a better understanding of the way in which genes can specify the development of the CNS and complex behaviours will be gained.

# Appendix

## Appendix 1

Appendix 1 contains courtship behaviour data from all individuals analyzed in this study (given individual numbers at the top of each column).

### CANTON S MALE

PARAMETER	1	2	3	4	5
CI	96.38	88.84	97.79	80.1	87.519
WEI	45.335	38.85	27.8	35.5	31.83
SI	44.812	26.69	23.61	30.154	24.875
WEI/CI	0.47037767	0.43730302	0.28428265	0.443196	0.36369246
SI/WEI	0.98846366	0.68700129	0.84928058	0.84940845	0.78149544
BOUITS	134	74	133	120	154
SECS	299.84	621	636.6	606.72	599.24
SBPM	26.814301	7.14975845	12.535344	11.8670886	15.4195314
MPPT	8	7	7	7	7
SECS	299.84	621	636.6	606.72	599.24
TRAINS	117	165	214	155	202
PTPM	23.4124867	15.942029	20.1696513	15.3283228	20.2256191
IPI (SEC)	0.032	0.033	0.031	0.031	0.033

PARAMETER	6	7	8	9	10
CI	96.6	91.073	82.1	99.528	97.895
WEI	29.78	41.358	24.5	37.407	61.871
SI	25.79	38.338	21.3	35.394	60.285
WEI/CI	0.30828157	0.45411922	0.29841657	0.37584398	0.63201389
SI/WEI	0.86601746	0.92697906	0.86938776	0.94618654	0.97436602
BOUITS	112	123	48	112	419
SECS	599.16	330.92	295.1	319.64	639.24
SBPM	11.215702	22.3014626	9.75940359	21.0236516	39.3279519
MPPT	6	9	9	8	8
SECS	599.16	330.92	295.1	319.64	639.24
TRAINS	219	135	63	102	354
PTPM	21.930703	24.477215	12.8092172	19.1465399	33.226957
IPI (SEC)	0.032	0.031	0.031	0.031	0.03

PARAMETER	11	12	13	14	15
CI	96.131	99.554	98.364	88.09	97.71
WEI	58.383	30.567	34.613	48.112	29.233
SI	42.333	29.528	29.063	47.484	27.572
WEI/CI	0.6073275	0.3070394	0.35188687	0.54616869	0.29918125
SI/WEI	0.72509121	0.96600909	0.83965562	0.98694712	0.94318065
BOUITS	139	100	83	121	81
SECS	302.74	300.04	301.12	300.48	341.88
SBPM	27.5483914	19.9973337	16.5382572	24.1613419	14.2155142
MPPT	9	8	9	8	10
SECS	302.74	300.04	301.12	300.48	341.88
TRAINS	139	86	89	82	82
PTPM	27.5483914	17.197707	17.7337938	16.3738019	14.3910144
IPI (SEC)	0.033	0.033	0.032	0.031	0.033

***fru<sup>M</sup>/Df(3R)fru<sup>4-40</sup> MALE***

PARAMETER	1	2	3	4	5
CI	96.77	98.2	94.93	95.77	97.35
WEI	34.9	61.03	49.48	51.32	51.11
SI	34.8	47.9	40	40.63	44.02
WEI/CI	0.36064896	0.62148676	0.52122617	0.53586718	0.52501284
SI/WEI	0.99713467	0.7848599	0.80840744	0.79169914	0.86127959
BOUITS	50	16	147	152	230
SECS	222	42	600.64	601.24	614.04
SBPM	13.5135135	22.8571429	14.6843367	15.1686515	22.4741059
MPPT	10	9	11	8	7
SECS	222	42	600.64	601.24	614.04
TRAINS	59	25	186	175	362
PTPM	15.9459459	35.7142857	18.5801811	17.4639079	35.3722885
IPI (SEC)	0.034	0.033	0.033	0.034	0.03

PARAMETER	6	7	8	9	10
CI	96.81	95.9	93.17	92.19	88.769
WEI	47.78	57.95	50.22	61.53	40.503
SI	40.874	57.01	47.54	55.43	33.39
WEI/CI	0.49354406	0.60427529	0.5390147	0.66742597	0.45627415
SI/WEI	0.85546254	0.98377912	0.94663481	0.90086137	0.82438338
BOUITS	217	299	187	313	172
SECS	593.12	599.8	600.16	702.2	595.48
SBPM	21.951713	29.90997	18.6950147	26.7445172	17.3305569
MPPT	8	8	9	7	8
SECS	593.12	599.8	600.16	702.2	595.48
TRAINS	376	434	313	399	268
PTPM	38.0361478	43.4144715	31.2916556	34.092851	27.0034258
IPI (SEC)	0.032	0.032	0.032	0.031	0.031

PARAMETER	11	12	13	14	15
CI	95.896	97.633	57.724	91.579	95.146
WEI	69.788	55.902	32.775	68.559	53.132
SI	63.39	54.655	31.805	68.269	50.902
WEI/CI	0.72774673	0.5725728	0.5677881	0.74863233	0.558426
SI/WEI	0.90832235	0.97769311	0.97040427	0.99577007	0.95802906
BOUITS	182	127	95	179	131
SECS	297.8	299.72	350	276.92	300.84
SBPM	36.6689053	25.4237288	16.2857143	38.7837643	26.1268448
MPPT	11	9	11	11	10
SECS	297.8	299.72	350	276.92	300.84
TRAINS	163	154	74	144	121
PTPM	32.8408328	30.8287735	12.6857143	31.2003467	24.1324292
IPI (SEC)	0.031	0.03	0.033	0.032	0.032

***fru<sup>Atra</sup> / Df(3R) fru<sup>4-40</sup> MALE***

PARAMETER	1	2	3	4	5
CI	96.28	95.8	92.648	97.97	92.28
WEI	62.95	39.9	33.021	48.85	46.024
SI	48.912	32.135	23.577	38.184	45.329
WEI/CI	0.65382219	0.41649269	0.35641352	0.49862203	0.49874296
SI/WEI	0.77699762	0.80538847	0.71400018	0.78165814	0.98489918
BOUITS	82	18	63	116	178
SECS	345.72	131.04	600.28	605.8	596.24
SBPM	14.2311697	8.24175824	6.29706137	11.4889402	17.9122501
MPPT	9	9	12	11	8
SECS	345.72	131.04	600.28	605.8	596.24
TRAINS	180	43	104	199	300
PTPM	31.2391531	19.6886447	10.3951489	19.7094751	30.1891856
IPI (SEC)	0.033	0.031	0.035	0.032	0.032

PARAMETER	6	7	8	9	10
CI	94.75	90.694	96.69	97.043	96.828
WEI	48.11	44.116	67.61	70.206	51.537
SI	45.93	36.33	45.06	56.513	47.087
WEI/CI	0.50775726	0.48642689	0.69924501	0.72345249	0.53225307
SI/WEI	0.95468718	0.82351074	0.66646946	0.80495969	0.91365427
BOUITS	184	187	217	168	103
SECS	600.04	641.44	599.36	307	293.2
SBPM	18.3987734	17.4918932	21.7231714	32.8338762	21.0777626
MPPT	9	8	7	9	11
SECS	600.04	641.44	599.36	307	293.2
TRAINS	302	323	408	233	123
PTPM	30.1979868	30.2132701	40.8435665	45.5374593	25.1705321
IPI (SEC)	0.033	0.028	0.034	0.031	0.031

PARAMETER	11	12	13	14	15
CI	88.63	94.605	94.579	99.2	96.99
WEI	44.412	49.684	49.63	60.789	71.988
SI	43.525	39.977	39.84	52.366	68.58
WEI/CI	0.50109444	0.52517309	0.52474651	0.61279234	0.74222085
SI/WEI	0.98002792	0.80462523	0.80274028	0.86143875	0.95265878
BOUITS	116	109	81	128	145
SECS	300.4	299.12	299.64	300	300.16
SBPM	23.1691079	21.8641348	16.2194634	25.6	28.9845416
MPPT	8	10	10	10	13
SECS	300.4	299.12	299.64	300	300.16
TRAINS	147	135	138	163	198
PTPM	29.3608522	27.079433	27.6331598	32.6	39.5788913
IPI (SEC)	0.033	0.033	0.031	0.029	0.03

*fru*<sup>Atra</sup> / *Df(3R)fru*<sup>4-40</sup> MALE

PARAMETER	16	17	18
CI	99.72	97.764	99.124
WEI	71.106	54.273	68.369
SI	62.781	53.89	66.356
WEI/CI	0.71305656	0.555143	0.68973205
SI/WEI	0.88292127	0.99294308	0.97055683
BOUTS	148	113	170
SECS	301.6	300.72	299.76
SBPM	29.4429708	22.5458899	34.0272218
MPPT	10	10	9
SECS	301.6	300.72	299.76
TRAINS	147	138	160
PTPM	29.2440318	27.5339186	32.0256205
IPI (SEC)	0.031	0.032	0.032

***fru<sup>M</sup>/Df(3R)fru<sup>4-40</sup>* FEMALE**

PARAMETER	1	2	3	4	5
CI	46.316	1.863	47.368	91.457	82.594
WEI	18.74	0.345	18.138	67.534	34.786
SI	5.05	0.066	7.746	34.747	9.201
WEI/CI	0.4046118	0.18518519	0.38291674	0.73842352	0.42116861
SI/WEI	0.26947705	0.19130435	0.42705921	0.51451121	0.26450296
BOUITS	0	0	0	0	0
SECS	323.2	301.56	329.84	300.84	325.56
SBPM	0	0	0	0	0
MPPT	3	3	3	3	3
SECS	323.2	301.56	329.84	300.84	325.56
TRAINS	16	1	14	100	22
PTPM	2.97029703	0.19896538	2.5466893	19.9441564	4.05455216
IPI (SEC)	0.024	0.029	0.029	0.027	0.028

PARAMETER	6	7	8	9	10
CI	75.283	24.964	83.966	15.133	59.827
WEI	53.97	7.245	35.289	4.367	27.659
SI	19.411	3.152	20.864	3.449	22.838
WEI/CI	0.71689492	0.29021791	0.42027726	0.28857464	0.46231635
SI/WEI	0.35966278	0.43505866	0.5912324	0.78978704	0.82569869
BOUITS	0	0	0	0	0
SECS	307.32	300.04	299.84	300.56	299.92
SBPM	0	0	0	0	0
MPPT	3	3	3	2	3
SECS	307.32	300.04	299.84	300.56	299.92
TRAINS	19	2	23	4	47
PTPM	3.70948848	0.39994667	4.60245464	0.79850945	9.40250734
IPI (SEC)	0.03	0.025	0.023	0.024	0.027

PARAMETER	11	12	13	14	15
CI	36.128	58.602	62.693	71.147	72.415
WEI	19.869	9.762	15.752	47.613	30.952
SI	9.83	1.589	1.019	28.261	3.638
WEI/CI	0.54996125	0.16658135	0.25125612	0.66922007	0.42742526
SI/WEI	0.49474055	0.16277402	0.0646902	0.59355638	0.11753683
BOUITS	0	0	0	0	0
SECS	301.84	300.6	300.2	299.52	302.96
SBPM	0	0	0	0	0
MPPT	2	2	3	3	3
SECS	301.84	300.6	300.2	299.52	302.96
TRAINS	3	3	2	44	7
PTPM	0.59634243	0.5988024	0.39973351	8.81410256	1.38632163
IPI (SEC)	0.021	0.029	0.026	0.029	0.028

***fru<sup>M</sup>/Df(3R)fru<sup>4-40</sup> FEMALE***

PARAMETER	16	17	18	19	20
CI	44.403	71.009	99.117	6.035	86.485
WEI	25.153	33.125	44.222	0.376	51.524
SI	7.707	8.782	19.675	0.032	25.453
WEI/CI	0.56647073	0.46649016	0.44615959	0.06230323	0.59575649
SI/WEI	0.3064048	0.26511698	0.4449143	0.08510638	0.49400279
BOUTS	0	0	0	0	0
SECS	302.36	310.52	298.32	311.44	311.56
SBPM	0	0	0	0	0
MPPT	4	3	3	0	3
SECS	302.36	310.52	298.32	311.44	311.56
TRAINS	20	17	106	0	65
PTPM	3.96877894	3.28481257	21.3193886	0	12.5176531
IPI (SEC)	0.03	0.026	0.027	0	0.023

PARAMETER	21	22	23	24	25
CI	81.004	75.584	54.219	65.889	95.274
WEI	27.184	47.214	12.565	20.013	36.2
SI	11.308	10.222	5.32	10.693	25.895
WEI/CI	0.33558837	0.62465601	0.23174533	0.3037381	0.37995676
SI/WEI	0.41597999	0.21650358	0.42339833	0.5343027	0.71533149
BOUTS	0	0	0	0	0
SECS	299.12	302.4	300.96	299.24	299.88
SBPM	0	0	0	0	0
MPPT	3	3		3	3
SECS	299.12	302.4	300.96	299.24	299.88
TRAINS	18	20	3	7	44
PTPM	3.61059107	3.96825397	0.59808612	1.40355567	8.80352141
IPI (SEC)	0.022	0.028	0.031	0.023	0.028

PARAMETER	26
CI	75.319
WEI	18.67
SI	9.858
WEI/CI	0.24787902
SI/WEI	0.52801285
BOUTS	0
SECS	300.16
SBPM	0
MPPT	3
SECS	300.16
TRAINS	16
PTPM	3.19829424
IPI (SEC)	0.025



*fru*<sup>Atra</sup> / *Df(3R)fru*<sup>4-40</sup> FEMALE

PARAMETER	1	2	3	4	5
CI	3.832	55.842	23.205	37.22	9.52
WEI	0	7.212	6.262	1.28	0
SI	0	0.559	3.911	0.537	0
WEI/CI	0	0.1291501	0.26985563	0.03439011	0
SI/WEI	0	0.07750971	0.62456084	0.41953125	0
BOUITS	0	0	0	0	0
SECS	291.08	321.92	290	301.64	300.76
SBPM	0	0	0	0	0
MPPT	0	2	3	1	0
SECS	291.08	321.92	290	301.64	300.76
TRAINS	0	1	19	1	0
PTPM	0	0.18638171	3.93103448	0.19891261	0
IPI (SEC)	0	0.023	0.02	0	0

PARAMETER	6	7	8	9	10
CI	72.133	35.672	33.337	7.64	62.821
WEI	22.532	2.693	10.516	0	16.362
SI	6.426	1.059	1.374	0	11.477
WEI/CI	0.31236743	0.07549338	0.3154453	0	0.26045431
SI/WEI	0.28519439	0.39324174	0.13065804	0	0.70144237
BOUITS	0	0	0	0	0
SECS	300.4	301.04	300.36	299.64	299.88
SBPM	0	0	0	0	0
MPPT	3	3	3	0	3
SECS	300.4	301.04	300.36	299.64	299.88
TRAINS	23	7	7	0	29
PTPM	4.59387483	1.39516343	1.39832201	0	5.80232093
IPI (SEC)	0.024	0.022	0.025	0	0.025

PARAMETER	11	12	13	14	15
CI	41.945	85.64	57.22	66.75	30.633
WEI	2.289	37.315	24.256	31.264	6.631
SI	0	17.909	18.64	5.965	3.443
WEI/CI	0.05457146	0.43571929	0.42390772	0.46837453	0.2164659
SI/WEI	0	0.47994104	0.76846966	0.19079452	0.51922787
BOUITS	0	0	0	0	0
SECS	299.52	297.88	301.56	300.24	300.16
SBPM	0	0	0	0	0
MPPT	2	3	3	3	2
SECS	299.52	297.88	301.56	300.24	300.16
TRAINS	1	28	72	17	6
PTPM	0.20032051	5.63985498	14.3255074	3.39728217	1.19936034
IPI (SEC)	0.021	0.025	0.023	0.025	0.03

$$fru^{Atra} / Df(3R) fru^{4-40} \text{ FEMALE}$$

PARAMETER	16	17	18	19	20
CI	29.595	62.49	71.314	85.929	86.011
WEI	3.117	21.87	29.564	45.325	48.904
SI	0.468	18.495	25.474	19.774	30.633
WEI/CI	0.10532184	0.349976	0.41456096	0.52747035	0.56857844
SI/WEI	0.15014437	0.84567901	0.86165607	0.43627137	0.62639048
BOUITS	0	0	0	0	0
SECS	300.12	301.04	300.48	298.2	300.2
SBPM	0	0	0	0	0
MPPT	2	3	3	3	3
SECS	300.12	301.04	300.48	298.2	300.2
TRAINS	2	45	64	26	104
PTPM	0.39984006	8.96890779	12.7795527	5.23138833	20.7861426
IPI (SEC)	0.03	0.022	0.023	0.025	0.026

PARAMETER	21	22	23	24	25
CI	4.343	61.034	61.228	62.163	9.273
WEI	0.245	7.332	5.302	20.002	0
SI	0	0.699	2.132	15.839	0
WEI/CI	0.05641262	0.12012976	0.08659437	0.32176697	0
SI/WEI	0	0.09533552	0.40211241	0.79187081	0
BOUITS	0	0	0	0	0
SECS	298	300.72	300.24	301	316.64
SBPM	0	0	0	0	0
MPPT	0	2	2	3	0
SECS	298	300.72	300.24	301	316.64
TRAINS	0	2	9	61	0
PTPM	0	0.3990423	1.79856115	12.1594684	0
IPI (SEC)	0	0.027	0.023	0.027	0

PARAMETER	26	27	28	29	30
CI	8.256	52.963	59.068	67.811	83.714
WEI	0.027	1.388	0.421	21.285	32.343
SI	0	0	0	8.132	12.356
WEI/CI	0.00327035	0.02620697	0.00712738	0.31388713	0.38635115
SI/WEI	0	0	0	0.38205309	0.38203011
BOUITS	0	0	0	0	0
SECS	296.44	299.32	300.56	301.12	300.32
SBPM	0	0	0	0	0
MPPT	0	0	0	2	3
SECS	296.44	299.32	300.56	301.12	300.32
TRAINS	0	0	0	19	26
PTPM	0	0	0	3.7858661	5.19445924
IPI (SEC)	0	0	0	0.022	0.028

*fru*<sup>Atra</sup> / *Df(3R)fru*<sup>4-40</sup> FEMALE

PARAMETER	31	32	33	34	35
CI	47.42	80.294	83.354	25.894	72.233
WEI	2.838	46.068	22.314	0	15.474
SI	0.968	18.647	4.845	0	0.742
WEI/CI	0.05984817	0.5737415	0.26770161	0	0.21422342
SI/WEI	0.34108527	0.40477121	0.21712826	0	0.0479514
BOUTS	0	0	0	0	0
SECS	300.76	299.32	299.96	300.48	298.44
SBPM	0	0	0	0	0
MPPT	2	3	3	0	3
SECS	300.76	299.32	299.96	300.48	298.44
TRAINS	3	70	15	0	3
PTPM	0.59848384	14.0318054	3.00040005	0	0.60313631
IPI (SEC)	0.016	0.024	0.026	0	0.036

PARAMETER	36	37	38	39	40
CI	73.652	64.002	74.15	85.1	68.597
WEI	35.112	29.291	7.051	28.749	13.909
SI	21.343	10.902	0	7.327	5.035
WEI/CI	0.4767284	0.45765757	0.09509103	0.33782609	0.20276397
SI/WEI	0.60785486	0.37219624	0	0.25486104	0.36199583
BOUTS	0	0	0	0	0
SECS	301.12	335.84	300.92	302.04	306.12
SBPM	0	0	0	0	0
MPPT	3	3	0	0	0
SECS	301.12	335.84	300.92	302.04	306.12
TRAINS	56	13	0	0	0
PTPM	11.1583422	2.32253454	0	0	0
IPI (SEC)	0.024	0.023	0	0	0

***tra<sup>1</sup>/Df(3L)st-J7 FEMALE***

PARAMETER	1	2	3	4	5
CI	92.094	98.64	94.183	95.859	99.324
WEI	46.884	53.672	45.163	58.627	26.183
SI	40.065	49.261	37.178	56.692	21.77
WEI/CI	0.50908854	0.54412003	0.47952391	0.6115962	0.26361202
SI/WEI	0.85455593	0.91781562	0.82319598	0.96699473	0.83145552
BOUITS	111	118	100	143	83
SECS	315	294.56	300.32	383.92	355
SBPM	21.1428571	24.0358501	19.9786894	22.3484059	32
MPPT	9	9	10	9	8
SECS	315	294.56	300.32	383.92	355
TRAINS	93	97	87	167	101
PTPM	17.7142857	19.7582835	17.3814598	26.0991873	17.0704225
IPI (SEC)	0.032	0.032	0.034	0.0035	0.034

PARAMETER	6	7	8	9	10
CI	96.524	95.324	96.84	98.765	95.684
WEI	47.512	46.324	48.795	49.678	45.389
SI	47.34	39.567	43.896	41.253	43.271
WEI/CI	0.49222991	0.48596366	0.50387237	0.50299195	0.47436353
SI/WEI	0.99637986	0.85413608	0.89960037	0.83040783	0.95333671
BOUITS	100	111	113	114	109
SECS	297.84	300.2	298.3	299.3	301.2
SBPM	20.1450443	22.1852099	22.7287965	22.8533244	21.7131474
MPPT	9	9	8	9	9
SECS	297.84	300.2	298.3	299.3	301.2
TRAINS	85	110	91	109	131
PTPM	17.1232877	21.9853431	18.3037211	21.8509856	26.0956175
IPI (SEC)	0.032	0.032	0.033	0.031	0.033

*In(3R)dsx<sup>23</sup>,fru<sup>3</sup>/Df(3R)dsx<sup>15</sup>/fru<sup>3</sup> MALE*

PARAMETER	1	2	3	4	5
CI	0	0	0	0	0
WEI	0	0	0	0	0
SI	0	0	0	0	0
WEI/CI	0	0	0	0	0
SI/WEI	0	0	0	0	0
BOUTS	0	0	0	0	0
SECS	0	0	0	0	0
SBPM	0	0	0	0	0
MPPT	0	0	0	0	0
SECS	0	0	0	0	0
TRAINS	0	0	0	0	0
PTPM	0	0	0	0	0
IPI (SEC)	0	0	0	0	0

PARAMETER	6	7	8	9	10
CI	0	0	0	0	0
WEI	0	0	0	0	0
SI	0	0	0	0	0
WEI/CI	0	0	0	0	0
SI/WEI	0	0	0	0	0
BOUTS	0	0	0	0	0
SECS	0	0	0	0	0
SBPM	0	0	0	0	0
MPPT	0	0	0	0	0
SECS	0	0	0	0	0
TRAINS	0	0	0	0	0
PTPM	0	0	0	0	0
IPI (SEC)	0	0	0	0	0

PARAMETER	11
CI	0
WEI	0
SI	0
WEI/CI	0
SI/WEI	0
BOUTS	0
SECS	0
SBPM	0
MPPT	0
SECS	0
TRAINS	0
PTPM	0
IPI (SEC)	0

## Appendix 2

Appendix 2 contains the individual data for the activity measurements taken from Canton S males and  $T\beta H^{nM18}$  octopamine mutant males.

### CANTON S MALE

PARAMETER	1	2	3	4
Line Crossings per Minute	29	26	25	32

PARAMETER	5	6	7	8
Line Crossings per Minute	25	30	28	20

PARAMETER	9
Line Crossings per Minute	30

### $T\beta H^{nM18}$ MALE

PARAMETER	1	2	3	4
Line Crossings per Minute	16	20	16	18

PARAMETER	5	6	7	8
Line Crossings per Minute	19	23	27	24

PARAMETER	9
Line Crossings per Minute	23

## Appendix 3

Appendix 3 contains the individual courtship behaviour and courtship song parameter measurements for individual Canton S males and  $T\beta H^{nM18}$  octopamine mutant males. The parameters analyzed are listed down the far left hand side, and the genotype at the top of the table.

### CANTON S MALE

PARAMETER	1	2	3	4	5
CI	82.022	93.937	77.219	90.666	86.605
WEI	56.518	72.188	40.351	46.724	63.389
SI	55.404	71.744	36.89	36.022	60.313
WEI/CI	0.68905903	0.76847249	0.52255274	0.51534202	0.73193234
SI/WEI	0.98028947	0.99384939	0.91422765	0.77095283	0.95147423
BOUTS	157	191	110	66	107
SECS	303.56	303.92	323.32	300.64	305.36
SBPM	31.0317565	37.7072914	20.4132129	13.1718999	21.0243647
MPPT	7	9	8	9	8
SECS	303.56	303.92	323.32	300.64	305.36
TRAINS	112	129	96	56	128
PTPM	22.137304	25.4672282	17.8151676	11.1761575	25.1506419
IPI (SEC)	0.031	0.031	0.031	0.033	0.031
CPP	1	1.5	1.5	1	2

PARAMETER	6
CI	92.575
WEI	55.124
SI	54.3358
WEI/CI	0.59545234
SI/WEI	0.98570133
BOUTS	110
SECS	325.76
SBPM	20.2603143
MPPT	7
SECS	325.76
TRAINS	106
PTPM	19.5235756
IPI (SEC)	0.032
CPP	1.5

***T<sub>BH</sub><sup>nM18</sup>* MALE**

PARAMETER	1	2	3	4	5
CI	74.134	88.345	82.464	79.54	58.9
WEI	33.384	32.982	30.179	33.291	15.145
SI	27.497	26.898	22.807	32.646	9.839
WEI/CI	0.45031969	0.37333182	0.36596575	0.41854413	0.25713073
SI/WEI	0.82365804	0.81553575	0.75572418	0.98062539	0.64965335
BOUITS	72	87	65	82	46
SECS	299.72	312.76	320.28	297.2	302.16
SBPM	14.4134526	16.6901138	12.1768453	16.5545087	9.13423352
MPPT	8	8	9	9	8
SECS	299.72	312.76	320.28	297.2	302.16
TRAINS	66	98	65	71	32
PTPM	13.2123315	18.8003581	12.1768453	14.333782	6.3542494
IPI (SEC)	0.035	0.034	0.035	0.035	0.034
CPP	1	2	1	1	1.5

PARAMETER	6	7	8	9	10
CI	53.392	93.785	86.513	85.04	87.253
WEI	22.137	32.501	39.642	40.78	39.263
SI	21.455	24.168	35.846	36.607	34.32
WEI/CI	0.41461268	0.34654796	0.45822015	0.47953904	0.44999026
SI/WEI	0.96919185	0.74360789	0.90424297	0.89767043	0.87410539
BOUITS	53	82	114	99	100
SECS	299.56	300.64	334.56	301.4	319.4
SBPM	10.6155695	16.3650878	20.4447633	19.7080292	18.7852223
MPPT	8	7	8	10	9
SECS	299.56	300.64	334.56	301.4	319.4
TRAINS	40	76	88	95	99
PTPM	8.01175057	15.1676424	15.7819225	18.9117452	18.5973701
IPI (SEC)	0.034	0.034	0.034	0.035	0.035
CPP	1	2	1	1.5	1

PARAMETER	11	12	13
CI	89.597	76.453	97.664
WEI	25.499	36.677	37.093
SI	20.083	35.567	30.975
WEI/CI	0.28459658	0.47973265	0.37980218
SI/WEI	0.78759951	0.9697358	0.83506322
BOUITS	57	24	96
SECS	308.04	330.96	283.56
SBPM	11.1024542	4.35097897	20.3131612
MPPT	8	9	8
SECS	308.04	330.96	283.56
TRAINS	82	25	91
PTPM	15.9719517	4.53226976	19.2551841
IPI (SEC)	0.034	0.036	0.035
CPP	1	1	1.5



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## Accompanying Materials